42

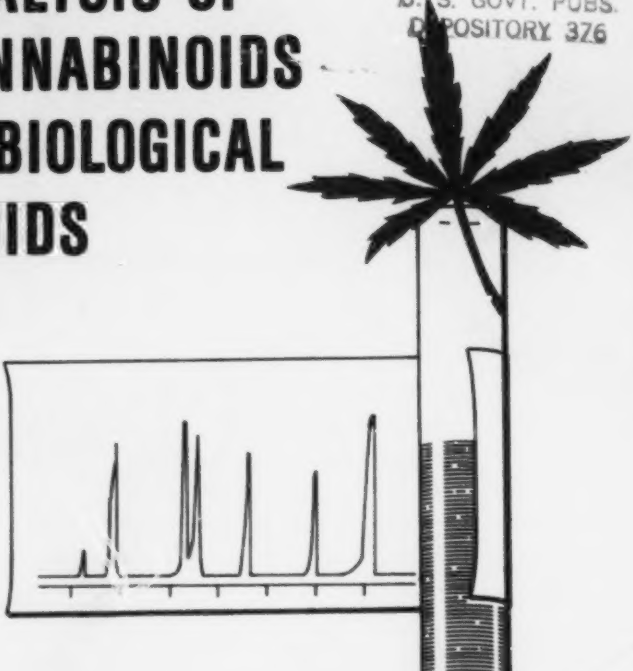
National
Institute on
Drug
Abuse

Research 42

MONOGRAPH SERIES

NEWARK PUBLIC LIBRARY
NOV 23 1982
U. S. GOVT. PUBS.
DEPOSITORY 376

THE ANALYSIS OF CANNABINOIDS IN BIOLOGICAL FLUIDS



U. S. DEPARTMENT OF HEALTH AND HUMAN SERVICES • Public Health Service • Alcohol, Drug Abuse, and Mental Health Administration

HE 20. 8216:

The Analysis of Cannabinoids in Biological Fluids

Editor:

Richard L. Hawks, Ph.D.
Research Technology Branch
Division of Research
National Institute on Drug Abuse

NIDA Research Monograph 42

1982

DEPARTMENT OF HEALTH AND HUMAN SERVICES
Public Health Service
Alcohol, Drug Abuse, and Mental Health Administration

National Institute on Drug Abuse
5600 Fishers Lane
Rockville, Maryland 20857

The NIDA Research Monograph series is prepared by the Division of Research of the National Institute on Drug Abuse. Its primary objective is to provide critical reviews of research problem areas and techniques, the content of state-of-the-art conferences, integrative research reviews and significant original research. Its dual publication emphasis is rapid and targeted dissemination to the scientific and professional community.

Editorial Advisory Board

Avram Goldstein, M.D.
Addiction Research Foundation
Palo Alto, California

Jerome Jaffe, M.D.
University of Connecticut School of Medicine
Farmington, Connecticut

Reese T. Jones, M.D.
Langley Porter Neuropsychiatric Institute
University of California
San Francisco, California

Jack Mendelson, M.D.
Alcohol and Drug Abuse Research Center
Harvard Medical School
McLean Hospital
Belmont, Massachusetts

Helen Nowlis, Ph.D.
Rochester, New York

Lee Robins, Ph.D.
Washington University School of Medicine
St. Louis, Missouri

NIDA Research Monograph Series

William Pollin, M.D.
DIRECTOR, NIDA
Marvin Snyder, Ph.D.
DIRECTOR, DIVISION OF RESEARCH, NIDA
EDITOR-IN-CHIEF
Eleanor W. Waldrop
MANAGING EDITOR

Parklawn Building, 5600 Fishers Lane, Rockville, Maryland 20857

The Analysis of Cannabinoids in Biological Fluids

ACKNOWLEDGMENT

This monograph is based on papers presented at a technical review held in Rockville, Maryland, on January 17, 1980, under the sponsorship of the Division of Research, National Institute on Drug Abuse.

COPYRIGHT STATUS

The material in this volume is in the public domain and may be used and reproduced without permission from the National Institute on Drug Abuse or the authors. Citation of the source is appreciated. Reproduction from the chapter by Moffat, Williams, and King requires acknowledgment that the material is under British Crown Copyright.

The United States Government does not endorse or favor any specific commercial product or commodity. Trade names or suppliers' names appearing in this publication are used only because they are considered essential in the context of the studies reported herein.

Library of Congress catalog card number 82-600586

DHHS publication number (ADM)82-1212
Printed 1982

NIDA Research Monographs are indexed in the *Index Medicus*. They are selectively included in the coverage of the *American Statistics Index*, *BioSciences Information Service*, *Chemical Abstracts*, *Current Contents*, *Psychological Abstracts*, and *Psychopharmacology Abstracts*.

FOREWORD

As the use of marijuana has increased over the last several years, the need for improved quantitative chemical assays with which to study the impact of marijuana use on the health of the individual has grown. More sensitive, reliable, and workable assays for cannabinoids (the active principles of marijuana) in body fluids are essential to increase our knowledge of how these chemical constituents and their metabolites accumulate and how they are eliminated from the body.

In addition to research requirements, there is a growing demand for less expensive and more reliable tests for use by the armed forces, civilian law enforcement agencies, and private industry where concern exists for the impact of marijuana use on critical job performance. Of particular concern are the effects of marijuana on complex performance related to driving motor vehicles, piloting aircraft, operating industrial equipment, monitoring radar screens, etc.

Concern about the negative impact of marijuana use on health has been elevated by recent research findings as well as the availability of more potent marijuana, the trend toward use among younger age groups, and the growing numbers of individuals with long-term exposure to marijuana. On the positive side of the health issue, tetrahydrocannabinol (THC), the primary active component of marijuana, is under intense investigation as a treatment for the often intolerable side effects of chemotherapy when other antiemetics are ineffective. Potential uses of THC for treatment of the symptoms of glaucoma, spasms related to muscular dystrophy, and certain asthmatic conditions are also under investigation. Research to assess both the clinical potential of THC and the effects of marijuana on health must rely in part on sensitive chemical analyses.

Differing methodologies for cannabinoid analysis are required to accommodate these various needs. Accordingly, the National Institute on Drug Abuse (NIDA) has supported a variety of efforts to develop sensitive cannabinoid assay methods by techniques of immunoassay, gas chromatography, gas chromatography/mass spectrometry, and high-performance liquid chromatography. The devel-

READEX MICROPRINT best copy available

opment of these methods was the subject of a technical review held by NIDA in February 1976 and described in NIDA Research Monograph 7. Since that time, research on techniques in the analysis of cannabinoids in biological fluids has been refined and new methods have been developed. Even more important, perhaps, has been the application of many of these methods to a wide variety of research problems.

In January 1980 NIDA held a second technical review to bring together key scientists working on the development of cannabinoid assays and to summarize recent research where cannabinoid analysis is being used. The present monograph presents this information as a compendium of the state of the art in the rapidly developing field of cannabinoid analysis research today.

It is our hope that this volume will be useful to basic researchers in the fields of biomedical and forensic science, where the availability of reliable, accurate, and accessible quantitative assays is critical to a better understanding of issues in marijuana abuse.

Marvin Snyder, Ph.D.
Director
Division of Research
National Institute on Drug Abuse

CONTENTS

Foreword
Marvin Snyder v

Introduction and Overview
Richard L. Hawks 1

The Use of High-Pressure Liquid Chromatography with Electrochemical Detection for the Assay of Nantradol and its Application to Δ^9-Tetrahydrocannabinol
Richard M. Shepard and George M. Milne 7

Radioimmunoassays for Cannabinoids
C. E. Cook, H. H. Seltzman, V. H. Schindler, C. R. Tallent, K. M. Chin, and C. G. Pitt 19

Radioimmunoanalysis of Delta-9-THC in Blood by Means of an ^{125}I Tracer
S. M. Owens, A. J. McBay, and H. M. Reisner 33

Significant Developments in Radioimmune Methods Applied to Δ^9-THC and its 9-Substituted Metabolites
J. R. Soares, J. D. Grant, and S. J. Gross 44

Combined High-Performance Liquid Chromatography and Radioimmunoassay Method for the Analysis of Δ^9-Tetrahydrocannabinol and its Metabolites in Plasma and Urine
Anthony C. Moffat, Peter L. Williams, and Laurence J. King 56

An EMIT Assay for Cannabinoid Metabolites in Urine
M. J. deLaurentis, K. McNeil, A. J. Mann, S. Clark, and Helena M. Greenwood 69

Laboratory Evaluation of Immunoassay Kits for the Detection of Cannabinoids in Biological Fluids
Michael A. Peat, Bryan S. Finkle, and Mary E. Deyman 85

Quantitative Analysis for Δ^9-THC, 11-Hydroxy-Δ^9-THC, and 9-Carboxy-Δ^9-THC in Plasma Using GC/CI-MS
Rodger L. Foltz and Bruce J. Hidy 99

Stability of Δ^9-Tetrahydrocannabinol in Stored Blood and Serum
Anthony S. Wong, Michael W. Orbanosky, Victor C. Reeve, and James D. Beede 119

The Constituents of Cannabis and the Disposition and Metabolism of Cannabinoids
Richard L. Hawks 125

List of Monographs 138

Analysis of Cannabinoids
Research Monograph 42, R. Hawks, Editor
National Institute on Drug Abuse, 1982

Introduction and Overview

Richard L. Hawks

The analysis of cannabinoids in biological fluids is a subject of considerable interest in several areas of public concern and also a subject of tremendous scientific challenge. Scientists involved in basic research on marijuana and its components are demanding more sensitive and accessible methods of quantitative analysis, civil enforcement organizations are interested in more effective ways of chemically determining intoxication or past use of marijuana, and professionals in the clinical field are becoming increasingly interested in tests to determine the extent of an individual's marijuana use habit in conjunction with treatment protocols.

In any area of assay development, and particularly in the drug abuse area, the questions of method sensitivity, specificity, and reliability are paramount. Another related but often overlooked area is that of drug pharmacokinetics, critical to the interpretation of a drug-concentration analysis.

The sensitivity of an assay is a measure of the least amount of material the assay will reliably detect. Because there are many ways to arrive at a number which represents this limit, the sensitivity claims for published assays do not always coincide with their practical working limits. When sensitivity is determined it is important to consider the type of body fluid involved and the way the sensitivity is determined. An assay which reliably detects one nanogram of a given drug in one milliliter of saline may not provide reliable detection at even 100 times that concentration in an actual serum or urine sample because of the interference of biological background noise. When determining the sensitivity of an assay it is important to prepare and analyze a significant number of blank body-fluid samples containing the same amount of analyte near the limit of sensitivity so that the precision of the assay can be statistically determined. The statistical variation of this "spiked" level should be compared with the variation observed in the blank samples to determine the probability of overlapping values. This is particularly important in assays designed for use as detection assays where there is concern for both false positive results and false negative results.

It is equally important to be able to distinguish the given drug of interest from other drugs, metabolites, and endogenous compounds that might appear in the sample matrix. The extent to which the assay can make this distinction is the specificity of the assay. This becomes particularly important in the case of forensic samples, where the qualitative analysis of the drug is often more important than the quantitative analysis. In the case of immunoassays, specificity is a particularly important factor. Historically, immunoassays have lacked high specificity because of the way antibody material was generated. As demonstrated in the papers presented by Drs. Cook and Soares, high specificity of radioimmunoassay (RIA) methods can be achieved by careful design and sophisticated techniques of synthesis in the preparation of the immunogens used to generate the reagent antisera. In some cases, however, an assay with high specificity for a specific parent drug or metabolite is not as desirable as one with limited cross-reactivity for a class of molecules, as in the case of the Syva homogeneous enzyme immunoassay (EMIT) presented by Drs. Greenwood and deLaurentis. Here cross-reactivity with a variety of Δ^9-tetrahydrocannabinol (THC) metabolites is desirable since the assay is used to determine past use of marijuana rather than a specific level of a given cannabinoid. By cross-reaction with more than a single metabolite, additional sensitivity is achieved.

Another analytical approach which benefits from the use of cross-reacting antisera is that which combines high-performance liquid chromatography (HPLC) with an RIA. This technique exploits the specificity to be achieved by HPLC chromatographic separation and the sensitivity possible by using an RIA as the detector for the system. A good example of this approach is described in the paper by Dr. Moffat.

Major advances since the time of the previous NIDA monograph on cannabinoid assays in 1976 have included the development of sensitive gas chromatography/mass spectrometry (GC/MS) methods for the detection of 11-hydroxy-THC and 11-nor-THC-9-carboxylic acid (9-carboxy-THC) at low-nanogram sensitivity. New achievements in immunoassays include the development of specific RIA methods for 9-carboxy-THC as well as the development of iodinated tracers which allow sensitivity levels near one nanogram per ml to be achieved. The demonstrated ability to use some of these assays for direct analysis of whole blood is also an important achievement for forensic research applications.

Detection of past use of marijuana could facilitate drug-treatment counseling and provide a broader base by which to assess the impact of marijuana on health and safety. This is of particular concern with respect to those groups which might be considered to be in relatively high-risk situations, either to themselves or others, and includes the young, members of the military, automobile drivers, and operators of complex industrial equipment. Systems like the Syva EMIT may provide some of this assessment in its role as a screening tool for particular populations.

In the case of drivers, equipment operators, and others whose level of sobriety is a key safety factor, the issue of an analytical means of determining intoxication is critical and a matter that has proven to be a much more difficult problem than mere detection. There are many specific factors to be considered when cannabinoid assays are used in the forensic area, including the convenience of obtaining the biological sample to be analyzed and the usual concerns of sensitivity, specificity, and other technical aspects of the assay. Interpretation of results in forensic cases becomes as important as the accuracy of the assay performed.

It appears from available data that only a blood sample may correlate physiologically with an actual state of intoxication. A urine sample which tests positive indicates prior use, but such an analysis cannot be directly related to intoxication since cannabinoid metabolites can persist in the urine for several days after smoking a single cigarette.

The use of saliva or breath for detection is very attractive because it involves an easily obtainable sample. Studies are in progress to further assess the potential of saliva or breath as a noninvasive alternative to blood collection in roadside surveys. Dr Soares' paper provides preliminary evidence that cannabinoids detectable by immunoassay techniques may be found in breath samples. While experiments involving intravenous injection of radiolabeled THC into human subjects with subsequent monitoring of radioactivity in saliva have indicated that negligible amounts of drug or metabolites cross the blood/saliva barrier, other experiments have shown that breath and blood samples collected after smoking marijuana do show detectable levels of THC which persist for several hours. These THC levels are apparently due to material sequestered directly, in a nonspecific way, in the mouth and lungs during smoking and then slowly released into breath or saliva. If simple methods for the analysis of these levels could be developed, it is conceivable that they could be used as a presumptive test of recent use of marijuana. Such a method of analysis would not, however, be quantitatively related to blood-level equivalents under these circumstances.

One of the most important issues in the area of marijuana and driving is the interpretation of a given blood concentration of THC to explain the presumptive intoxication of the individual from whom the sample was taken. In an attempt to determine whether it is feasible to establish a presumptive level of impairment for THC, studies were initiated under NIDA contract at the Southern California Research Institute to study the effects of various doses of marijuana on behavioral tasks related to driving ability. Coincident with the behavioral study, a pharmacokinetic analysis of each subject was performed. These studies were designed to generate two sets of time-course data–one of blood concentrations and one of behavioral performance. The sensitive behavioral tests were carried out periodically to provide a performance time-course curve which could be statistically compared to the pharmacokinetic blood-level curve. Analysis of this data is not yet complete, but what is obvious so far is that even though some consistency exists across individuals smoking a given dose of marijuana, in terms of expected blood levels, the associated performance effects of these doses do not show the same consistency. It is not yet clear whether a practical presumptive concentration of THC can be related to measurable impairment.

Research on the therapeutic use of THC is a rapidly developing area. Large State and national programs have been started to investigate the therapeutic potential of THC as an antiemetic for chemotherapy patients. Most of this research involves oral formulations of THC in sesame oil capsules. The chapter on metabolism and disposition indicates that the oral route of administration produces a relatively large amount of 11-hydroxy-THC, which is a pharmacologically active metabolite, and an assay suitable for this type of clinical research must be designed to determine both THC and 11-hydroxy-THC levels. At the present time only the GC/MS method and methods which couple HPLC with RIA can be applied to this type of analysis. It is expected however that a specific RIA method being tested at the Research Triangle Institute will be available soon for separately determining 11-hydroxy THC in the presence of THC.

There has been increasing interest in the use of nonspecific RIA's in combination with the chromatographic separation properties of HPLC instruments. The paper by Dr. Moffat demonstrates one particular use of this system in the area of cannabinoid analyses. The combination of these two methods constitutes a powerful research tool in that it enables the investigator to determine quantitatively with a single radioimmunoassay the concentrations of a large number of metabolites. The tradeoff is a somewhat longer time of analysis, but in a research setting or with a small number of samples, time is not often a major factor. HPLC has also been used as an integral part of the sample preparation procedure for GC/MS analyses, as noted by Dr. Foltz. Drs. Shepard and Milne report a method of reasonable sensitivity with use of HPLC and standard detectors.

Dr. Moffat, in forensic cases examined by his group, reports significant amounts of glucuronide-conjugated metabolites in urine and blood. Dr. Peat reported that a large number of EMIT-positive urine samples, when subsequently analyzed by GC/MS, showed levels that were low or undetectable for free 9-carboxy-THC. In view of Dr. Moffat's comments, further analysis of some of these samples (after the technical review) was carried out. In a number of cases, samples previously low or negative for 9-carboxy-THC showed significant levels after enzymatic hydrolysis. This finding indicates that in some urine samples virtually all of the 9-carboxy-THC may be found in a conjugated form, an important factor when a specific method such as GC/MS is used as a confirmation tool for a less specific assay such as the EMIT, which probably cross-reacts with the glucuronide metabolite. This hydrolysis technique has been applied to the confirmation of a large number of EMIT-positive samples resulting from a Department of Defense screening study. The Armed Forces Institute of Pathology used GC/MS to confirm over 95 percent of 1100 positive urine samples resulting from the study. Most of the questionable positive results which made up the 5 percent unconfirmed cases were close to the cutoff level of detection of the EMIT assay.

Dr. Owens reported that his highly specific and sensitive RIA worked quite well with a methanol extract of whole blood. Previously, many investigators assumed that, for an immunoassay to function properly, the medium in which the antigen-antibody interaction occurred would have to be quite similar to a normal physiological environment. It appears, however, that at least in the case of the cannabinoid RIAs, this is not necessarily the case. In fact the assay was shown to work better in the presence of alcohol than in its absence. This also had been the experience of Drs. Moffat and Cook, an important finding because it enables a blood sample to be partially purified by alcohol precipitation, centrifugation, and subsequent analysis of the resulting alcoholic supernatant without an evaporation step. The presence of alcohol may facilitate the assay by reducing "sticking" of THC to nonspecific binding sites.

Of interest to the forensic community are the comments of Dr. Wong concerning his studies on the stability of cannabinoids in whole blood and plasma under different conditions. These studies were carried out under contract to the Department of Justice, State of California, as part of that State's research efforts to provide a basis for future legislation concerning marijuana and driving impairment.

Interpretation of data from forensic samples was an issue of considerable discussion at this technical review. Because THC has a relatively short half-life after smoking, assay method sensitivity of 1 ng per ml may not be sufficient to detect the compound more than 6 hours after smoking. This has led some investigators to argue that if any level of THC is detectable in blood at all, one can assume that the sample was taken at a time very close to the incident of smoking and that, therefore, the subject has a high probability of being intoxicated. This argument makes fairly broad assumptions concerning the presumptive concentrations of THC that can be related to specific types of intoxicated behavior. Quite obviously we are still a long way from determining exactly what this presumptive level is. It was generally agreed, however, that for any THC blood assay to be of use in the forensic area, it would have to have a sensitivity of at least 3 nanograms per ml since blood levels fall to this level within an hour or two after a smoking incident. While little information is currently available on THC blood levels in chronic smokers, the possibility of accumulated drug being at detectable levels long after the acute period of intoxication must be considered.

While it is obvious that considerable progress has been made in the last few years on cannabinoid assays, research in this area continues to develop at a rapid pace. From the presentations and discussions at this meeting, it seems that the immediate future holds interesting breakthroughs in even more sensitive assays as well as improvements in methods which will lead to cannabinoid assays being used for the first time in a variety of new forensic and social settings.

AUTHOR

Richard L. Hawks
Research Technology Branch
Division of Research
National Institute on Drug Abuse
Rockville, Maryland 20857

Analysis of Cannabinoids
Research Monograph 42, R. Hawks, Editor
National Institute on Drug Abuse, 1982

The Use of High-Pressure Liquid Chromatography with Electrochemical Detection for the Assay of Nantradol and its Application to Δ^9-Tetrahydrocannabinol

Richard M. Shepard and George M. Milne

High-pressure liquid chromatography with electrochemical detection permits sensitive, selective and quantitative analysis of biological and pharmaceutical compounds containing electroactive functional groups. In support of pharmacological and clinical studies with nantradol, a potent, non-opioid analgetic, a specific HPLC/electrochemical assay for nantradol and related compounds in plasma was recently developed. In preliminary studies we have now extended this technique to the detection of Δ^9- and 11-OH-Δ^9-tetrahydrocannabinol. Results are presented which indicate that HPLC coupled to an electrochemical detector provides sensitivity for these compounds in plasma in the low ng/ml range.

INTRODUCTION

High-performance liquid chromatography (HPLC) is finding increasing application to the trace analysis of drugs, drug metabolites and biologically important compounds in complicated matrices such as body fluids and tissue samples. In the case of cannabinoids, HPLC has been used as a means of purifying Δ^9-tetrahydrocannabinol (Δ^9-THC) from related materials and interfering endogenous materials in blood plasma. However, at the concentrations of Δ^9-THC normally encountered in plasma, the direct HPLC on-line monitoring methods such as refractive index or ultraviolet spectrophotometry detection are not sensitive enough for use in plasma level monitoring. As a result, subsequent analysis by gas chromatography (1), mass spectrometry (2), or radioimmunoassay (3) has been required. The coupling of electrochemical detection to HPLC (4) is a recently developed technique that often provides the sensitivity and selectivity necessary for practical analytical procedures. The application of this technology to the determination in plasma of nantradol, a potent analgetic, has recently been described (5). It is the aim of this paper to describe briefly the principles of electrochemical detection linked to HPLC, to examine the application of this technique to the analysis of nantradol in plasma, and then to discuss efforts to extend this method to the determination of Δ^9-THC in plasma.

PRINCIPLES OF HPLC COUPLED WITH ELECTROCHEMICAL DETECTION

In HPLC with electrochemical detection (figure 1), material eluted from the chromatographic column reacts at an electrode surface under controlled potential conditions, and the current which results from the net exchange of electrons is monitored as a function of time (6). Since the amount of material converted by the electrochemical reaction is proportional to the instantaneous concentration, the current will be directly related to the amount of compound eluted as a function of time. If chromatographic conditions are carefully controlled, then amperometric detection is quite precise, with a wide linear range of typically 10^6, and quantitative data can be obtained at the picomole level for many compounds. Limits of detection down to 1 pg or less are attainable in some cases.

In addition to being extremely sensitive, the electrochemical detector is selective in that only compounds containing an electrochemically oxidizable or reducible functional group can be detected (6). Furthermore, these compounds must be electroactive at the given potential to which the detector is set. While these features represent both an advantage and disadvantage, they often combine to make sample preparation a relatively simple matter.

With direct conversion of a chemical signal into an electrical signal, the electronic instrumentation of the electrochemical detector is therefore inexpensive and reliable. The detector cell is well suited for HPLC analysis because it has an extremely low dead volume. Because of the nature of electrochemical detection, the

Picomole sensitivity

Selective

Low cost

Can be combined with HPLC

Used in analysis of:
- **phenols**
- **aromatic amines**
- **catecholamines**
- **antioxidants**
- **ascorbic acid**
- **uric acid**
- **natural products**
- **pharmaceuticals**

FIGURE 1. Features and applications of electrochemical detection.

chromatographic systems most promising for this type of detector are ion-exchange and reversed-phase systems where aqueous or aqueous-alcohol solvent mixtures containing some dissolved ions can be used (6).

A number of endogenous compounds, drugs, and drug metabolites have been studied by HPLC with electrochemical detection. Examples include phenols (7), aromatic amines (6,8), catecholamines (9,10,11), ascorbic acid (12) and uric acid (13). This methodology has found increasing application in the field of neurochemical analysis, having been used for the simultaneous determination, in rat and mouse brains, of nanogram levels of catecholaminergic and serotonergic neurotransmitters such as norephinephrine, dopamine, tryptophan and serotonin (14,15).

APPLICATION OF HPLC AND ELECTROCHEMICAL DETECTION TO NANTRADOL

Nantradol (figure 2) produces analgetic activity 2 to 7 times greater than morphine across a range of analgetic tests traditionally responsive only to the narcotic agonist analgetics, but it does not bind to the opiate receptor *in vitro* (16). While nantradol possesses certain features of cannabinoid pharmacology, it has a number of structural features which distinguish it from the cannabinoids–including the absence of a pyran oxygen, the presence of a weakly basic nitrogen and the introduction of an oxygen-containing side chain. *In vivo* nantradol is rapidly deacetylated to the free phenol, desacetylnantradol, which is the only form of the drug detectable in plasma (figure 2). The phenol function, which is shared with Δ^9-THC, makes possible the electrochemical detection of desacetylnantradol.

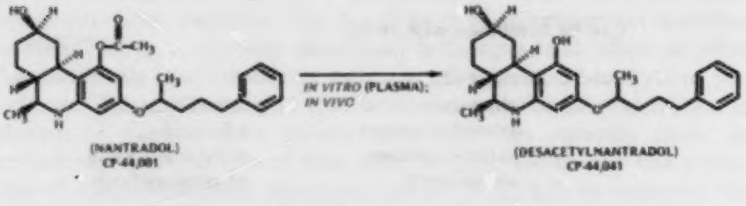
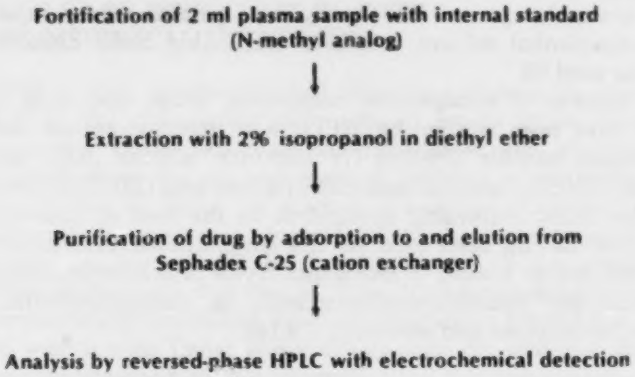


FIGURE 2. Structure of nantradol (CP-44,001), a potent non-opioid analgetic, and its deacetylation to desacetylnantradol (CP-44,041).



Fortification of 2 ml plasma sample with internal standard (N-methyl analog)

↓

Extraction with 2% isopropanol in diethyl ether

↓

Purification of drug by adsorption to and elution from Sephadex C-25 (cation exchanger)

↓

Analysis by reversed-phase HPLC with electrochemical detection

FIGURE 3. Flow scheme outlining the procedure for the analysis of desacetylnantradol in plasma.

The assay procedure (figure 3) begins with the fortification of a 2 ml plasma sample with 20 ng/ml of an internal standard, the N-methyl analog of desacetylnantradol (5,17). Extraction of the drug and internal standard is achieved with 2% isopropanol in diethyl ether. The extract is added to 2 ml of methanol and concentrated under nitrogen to a final volume of 1 ml. The extracted drug and internal standard are then adsorbed onto cation exchanger Sephadex C-25 (added in 1 ml as a 30% suspension in water), collected by centrifugation and the supernatant discarded. Following a water wash, the desacetylnantradol and internal standard are eluted from the Sephadex C-25 with 1 ml of acetonitrile:0.05 M KH_2PO_4 (1:1). Analysis is achieved by injection of 50 μl of the assay solution onto a reversed-phase HPLC Waters μBondapak phenyl column eluted with a solvent system of acetonitrile:0.05 M KH_2PO_4(1:1) at a flow rate of 2.0 ml/min. Two typical HPLC chromatograms of the analysis of desacetylnantradol isolated from human plasma samples fortified with 20 ng/ml of internal standard and either 5 or 20 ng/ml of drug are shown in figure 4. Unknown drug levels are first expressed as the ratio of the peak height corresponding to desacetylnantradol versus that for 20 mg/ml of the internal standard. Concentrations are then determined from an appropriately constructed standard curve (figure 5), in which known amounts of drug have been added to plasma. Good linearity is obtained and the precision of the assay is about 10% relative standard deviation based on the mean normalized peak height ratios within an assay series. The sensitivity of the assay permits the analysis of desacetylnantradol in plasma down to levels of about 5 ng/ml. As little as 200 pg of drug injected on-column can be detected.

APPLICATION OF HPLC/ELECTROCHEMICAL DETECTION TO Δ^9-THC

In light of the common presence of a C-1 phenol in desacetylnantradol and Δ^9-THC, we have carried out some initial experiments designed to investigate the feasibility of analyzing Δ^9-THC using HPLC with electrochemical detection. The HPLC system for the analysis of nantradol was adapted to Δ^9-THC by using the same reversed-phase column and detector potential and is probably not optimal. A less polar solvent of 55:45 acetonitrile:0.05 M KH_2PO_4 at a flow rate of 2.0 ml/min was used. In this system Δ^9-THC and 11-hydroxy-Δ^9-tetrahydrocannabinol (11-OH-Δ^9-THC), are readily separated from each other (figure 6), eluting at 16.6 ml and 8.2 ml, respectively. As little as 0.7 ng of pure Δ^9-THC injected on-column can be detected in this system. When various amounts of pure Δ^9-THC, from 0.7 ng to 154 ng, are injected onto the column the resulting peak response curve is linear over the entire range with a correlation coefficient of 0.999 and a precision of 6.3% relative standard deviation based on the mean normalized peak heights (figure 7).

In preliminary attempts to determine Δ^9-THC levels in plasma, dog plasma (2 ml) was extracted three times with hexane (3 ml). The pooled hexane extracts were washed 3 times with 3% KOH in

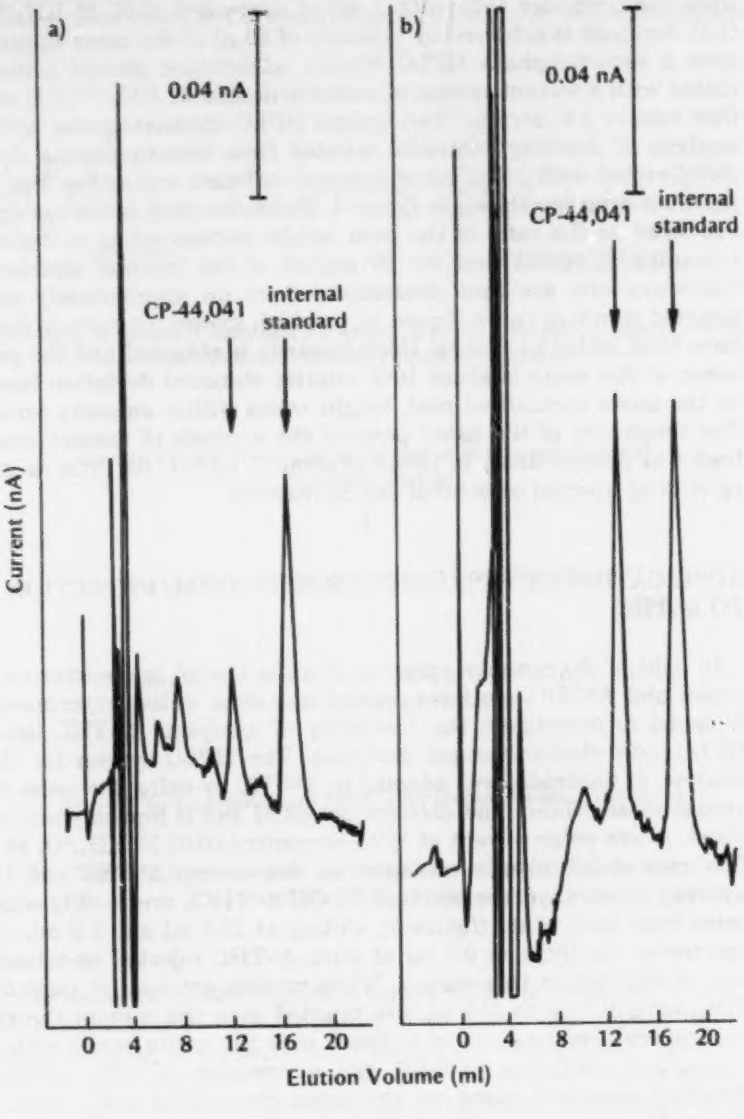


FIGURE 4. HPLC chromatograms of desacetylnantradol and internal standard isolated from human plasma, fortified with 20 ng/ml of N-methyl-desacetylnantradol and either a) 5 ng/ml or b) 20 ng/ml of desacetylnantradol.

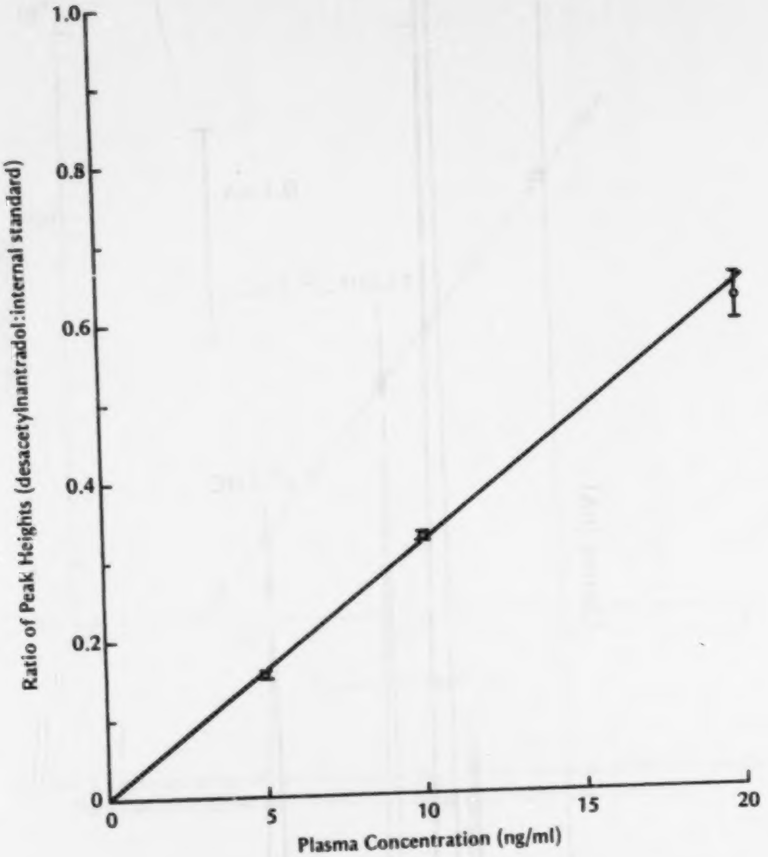


FIGURE 5. Standard curve illustrating the changes in the ratio of the peak heights of desacetylnantradol versus internal standard as a function of varying concentrations of desacetylnantradol in fortified human plasma.

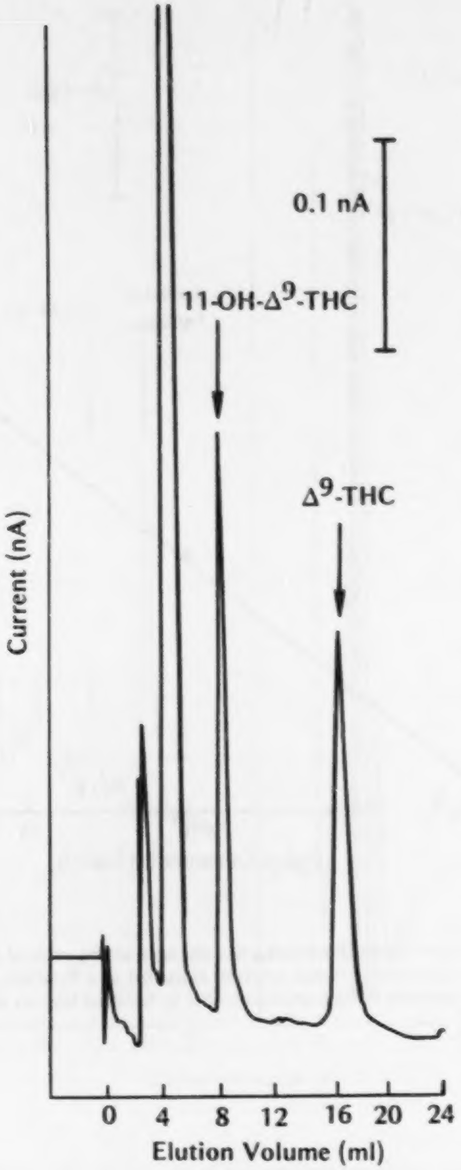


FIGURE 6. HPLC chromatogram illustrating the detection and separation of Δ^9-THC and 11-OH-Δ^9-THC.

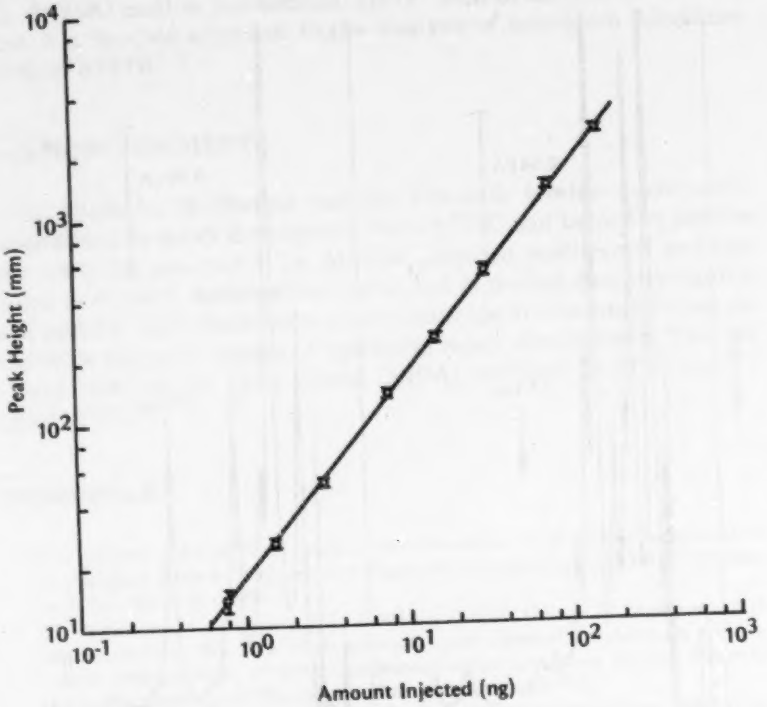


FIGURE 7. Demonstration of the linearity of peak height response of Δ^9-THC versus the amount injected onto the HPLC, using electrochemical detection.

20% aqueous methanol. The pooled alkali extracts were acidified with concentrated HCl, diluted with water and back-extracted with hexane. This hexane extract was then evaporated under nitrogen and the residue was redissolved in 200 μl of HPLC solvent. 100 μl of the solution was then injected onto the column. The HPLC profiles obtained from blank dog plasma and dog plasma fortified with 20 ng/ml Δ^9-THC are shown in figure 8. The average recovery of Δ^9-THC in three samples was 38% ± 2%. Based on these results, the limit of detection for the plasma determination of Δ^9-THC by this procedure is approximately 5 ng/ml.

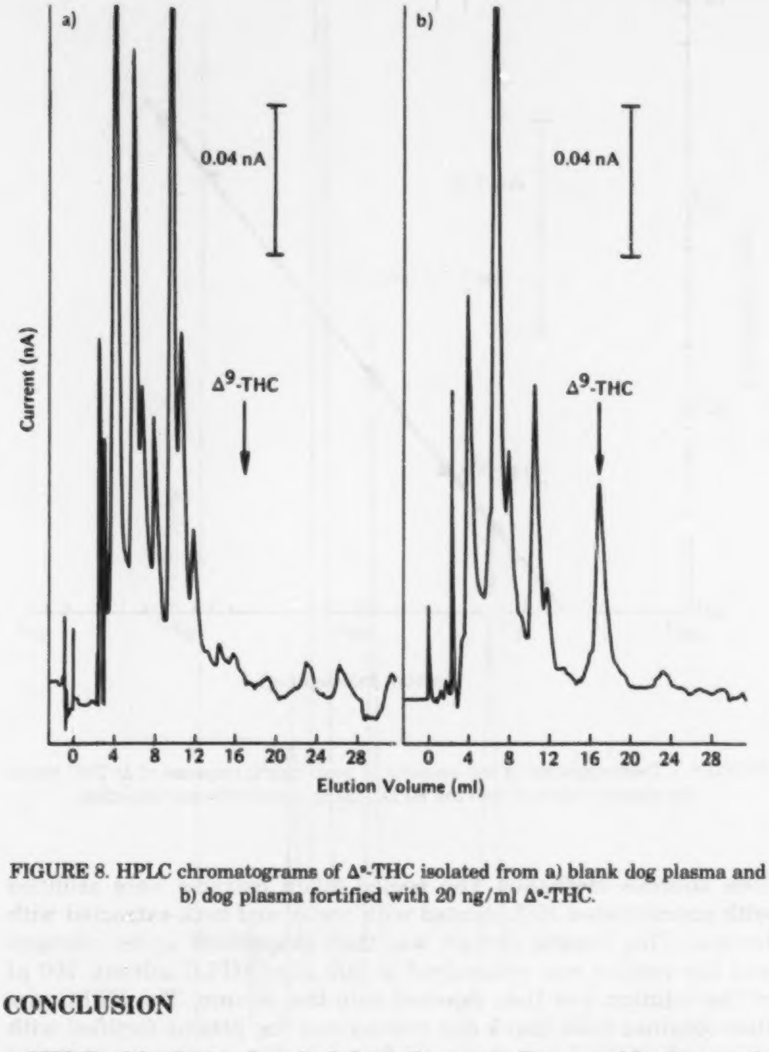


FIGURE 8. HPLC chromatograms of Δ^9-THC isolated from a) blank dog plasma and b) dog plasma fortified with 20 ng/ml Δ^9-THC.

CONCLUSION

HPLC with electrochemical detection can often provide the sensitivity and selectivity necessary for practical analytical procedures, as in the case of nantradol. It has been demonstrated here that this methodology is capable of detecting low nanogram levels of Δ^9-THC in plasma while providing a separation of Δ^9-THC from its major metabolite 11-OH-Δ^9-THC. Thus it appears that, while further development of this technique is needed for a routine plasma assay for Δ^9-THC and its metabolites, HPLC with electrochemical detection is a feasible approach to the analysis of nanogram concentrations of Δ^9-THC.

ACKNOWLEDGMENTS

Dr. Hugh M. McIlhenny and Mr. Frank R. Mosher made useful suggestions in assay development for Δ^9-THC and helped to develop the assay for nantradol. Dr. M. Ross Johnson synthesized and provided nantradol, desacetylnantradol and N-methyl-desacetylnantradol, and Dr. Carl Hausmeyer made available an electrochemical detector in the early stages of nantradol assay development. The National Institute on Drug Abuse (NIDA) provided Δ^9-THC and 11-OH-Δ^9-THC.

REFERENCES

1. E.R. Garrett and C.A. Hunt. Separation and analysis of Δ^9-tetrahydrocannabinol in biological fluids by high-pressure liquid chromatography and GLC. *J. Pharmaceut. Sci.*, *66*, 20-26 (1977).
2. J.L. Valentine, P.J. Bryant, P.L. Gutshall, O.H.M. Gan, P.D. Lovegreen, E.D. Thompson, and H.C. Niu. High-pressure liquid chromatographic-mass spectrometric determination of Δ^9-tetrahydrocannabinol in human plasma following marijuana smoking. *J. Pharmaceut. Sci.*, *66*, 1263-1266 (1977).
3. P.L. Williams, A.C. Moffat, and L.J. King. Combined high-pressure liquid chromatography and radioimmunoassay method for the quantitation of Δ^9-tetrahydrocannabinol and some of its metabolites in human plasma. *J. Chromatog.*, *155*, 273-283 (1978).
4. P.T. Kissinger, C. Refshauge, R. Dreiling, and R.N. Adams. An electrochemical detector for liquid chromatography with picogram sensitivity. *Anal. Lett.*, *6*, 465-477 (1973).
5. R.W. Mast, H.M. McIlhenny, G.M. Milne, and M.R. Johnson. Behavioral effects and pharmacokinetics of nantradol hydrochloride, a potent, cannabinoid-related analgesic. *Pharmacologist*, *21*, 270 (1979).
6. P.T. Kissinger. Amperometric and coulometric detectors for high-performance liquid chromatography. *Anal. Chem.*, *49*, 447A-456A (1977).
7. R.M. Riggin, L.D. Rau, R.L. Alcorn, and P.T. Kissinger. Determination of phenolic sympathetic stimulants in pharmaceuticals by liquid chromatography with electrochemical detection. *Anal. Lett.*, *7*, 791-798 (1974).
8. L.A. Sternson and W.J. DeWitte. High-pressure liquid chromatographic analysis of isomeric aminophenols with electrochemical detection. *J. Chromatog.*, *138*, 229-231 (1977).
9. R.M. Riggin, R.L. Alcorn, and P.T. Kissinger. Liquid chromatographic method for monitoring therapeutic concentrations of L-dopa and dopamine in serum. *Clin. Chem.*, *22*, 782-784 (1976).

10. P.T. Kissinger, R.M. Riggin, R.L. Alcorn, and L.D. Rau. Estimation of catecholamines in urine by high-performance liquid chromatography with electrochemical detection. *Biochem. Med.*, *13*, 299-306 (1975).
11. C. Refshauge, P.T. Kissinger, R. Dreiling, C.L. Blank, R. Freeman, and R.N. Adams. New high-performance liquid chromatographic analysis of brain catecholamines. *Life Sciences*, *14*, 311-322 (1974).
12. L.A. Pachla and P.T. Kissinger. Determination of ascorbic acid in foodstuffs, pharmaceuticals, and body fluids by liquid chromatography with electrochemical detection. *Anal. Chem.*, *48*, 364-367 (1976).
13. L.A. Pachla and P.T. Kissinger. Estimation of serum uric acid by high-performance liquid chromatography with electrochemical detection. *Clin. Chim. Acta*, *59*, 309-312 (1975).
14. S. Sasa and C.L. Blank. Simultaneous determination of norephinephrine, dopamine and serotonin in brain tissue by high-pressure liquid chromatography with electrochemical detection. *Anal. Chim. Acta*, *104*, 29-45 (1979).
15. I.V. Mefford and J.D. Barchas. Determination of tryptophan and metabolites in rat brain and pineal tissue by reversed-phase high-performance liquid chromatography with electrochemical detection. *J. Chromat.*, *181*, 187-193 (1980).
16. G.M. Milne, A. Weissman, B.K. Koe, and M.R. Johnson. CP-44,001, A novel benzo(c)quinoline analgesic. *Pharmacologist*, *20*, 243 (1978).
17. H.M. McIlhenny, R.W. Mast, M.R. Johnson, and G.M. Milne. Nantradol hydrochloride: Pharmacokinetics and behavioral effects following acute and chronic treatment. *J. Pharmacol. Exp. Ther.*, *219* (2), 363-369 (1981).

AUTHORS

Richard M. Shepherd and George M. Milne
Pfizer Research Laboratories
Groton, Conn. 06340

Analysis of Cannabinoids
Research Monograph 42, R. Hawks, Editor
National Institute on Drug Abuse, 1982

Radioimmunoassays for Cannabinoids

C. E. Cook, H. H. Seltzman, V. H. Schindler, C. R. Tallent, K. M. Chin, and C. G. Pitt

The simplicity, sensitivity, and specificity of radioimmunoassay have made it an attractive procedure for the analysis of delta-9-tetrahydrocannabinol (THC) in biological fluids or tissues. The presence of closely related compounds such as metabolites may interfere with radioimmunoassay results. Appropriate design of immunogens may diminish such interference. This work has been directed towards the use of the amyl side chain for linking cannabinoid compounds to proteins to form immunogens. Although the amyl side chain is metabolized to some extent, the metabolites are not quantitatively significant in most cases. 5'-Carboxy-delta-8-THC and 5'-carboxy-delta-9-THC were linked to bovine serum albumin. Immunization of rabbits with the resulting conjugates resulted in the formation of antisera with high selectivity for delta-9-THC vs. its carboxylic acid metabolite, 11-nor-9-carboxy-delta-9-THC. Delta-8-THC radioligands (4',5'-tritium and 5'-iodine-125) could be used with these antisera for analysis of delta-9-THC in plasma. Sensitivity with tritium-labeled material is about 2.5 ng/ml. 5'-Oxo-11-nor-9-carboxy-delta-8-THC was used to prepare an immunogen which led to the generation of an antiserum highly specific for 11-nor-9-carboxy-delta-9-THC. This antiserum and iodine-125-5'-iodo-11-nor-9-carboxy-delta-8-THC were used to develop a highly specific assay for 11-nor-9-carboxy-delta-9-THC in plasma.

INTRODUCTION

The basic premise of immunoassay techniques is that a labeled antigen will interact with a specific antibody to form a labeled antigen/antibody complex. In the presence of unlabeled antigen there will be a competition for binding sites to the antibody, thereby decreasing the amount of the complex which is labeled. In order to achieve a useful assay, then, one must be able to obtain a labeled antigen, to generate a specific antibody which will bind the compound of interest, and to develop some means of distinguishing the labeled complex from the free labeled antigen.

In order to stimulate the formation of antibodies which will selectively bind a small molecule such as tetrahydrocannabinol, the molecule or hapten must first be linked to a large molecule, generally a protein (figure 1). This concept was demonstrated by Landsteiner many years ago (1). Landsteiner also showed that the position of the linkage was of considerable importance in determining the selectivity of the resulting antibody. The fit between antibody and antigen is often quite good, particularly in areas which are removed from the site of attachment to the protein. However, introduction of the covalent link inevitably results in a change in the structural characteristics of the molecule. The resulting antibody often reflects this by being insensitive to changes around the position of linkage, as is illustrated by the hatched area in figure 1.

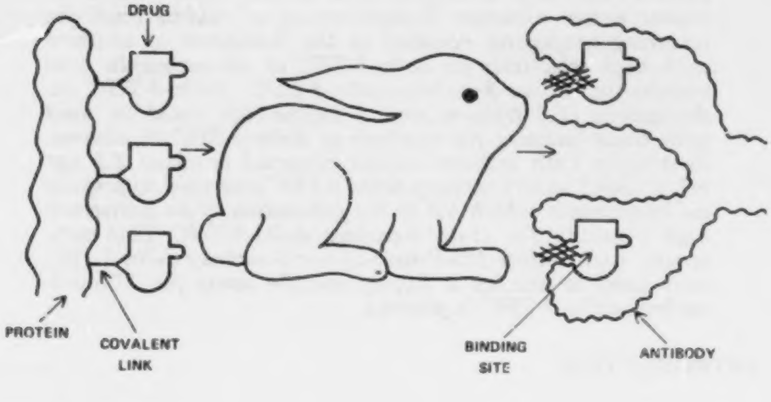


FIGURE 1. Generation of antibodies to a drug. The drug (hapten component) is covalently linked to a large molecule such as a protein to form the immunogen. Injection of this into animals stimulates formation of antibodies. The binding site of the antibody accommodates the hapten, with some ambiguity around the area which corresponds to the point of linkage in the immunogen.

Since in general a drug and its metabolites will have many similar structural features, the metabolites will usually be the principal interfering constituents in an RIA analysis. Thus, in preparing a conjugate to generate specific antisera, one tries to avoid sites on the drug which are metabolically reactive, to leave them free to influence the formation of selective antibodies. Also one tries to avoid covering up structural features which may permit development of strong affinity binding sites. In addition, one must also consider the chemistry involved and its difficulty in relation to the expected benefit.

It is now generally accepted that the principal metabolic alterations in the THC molecule occur in the 8- and 11-positions. Hydroxylation may occur in these positions, and oxidation to an 11-nor-9-carboxy metabolite is also a principal pathway. Metabolites involving the amyl side chain have also been reported, but it appears that in quantitative terms these are relatively minor--at least in humans (2).

Nonselective antisera are also useful, but our interest has been in the development of assays with specificity for one compound. Specificity of an assay may be increased by techniques such as differential extraction of the desired compound or addition of a second antibody to tie up the interfering substance (3). However, these techniques work better if the antiserum has good selectivity to begin with. We therefore chose to examine immunogens formed by attaching cannabinoid moieties to proteins through the amyl side chain, as this would fulfill our requirement of distance from the principal sites of metabolism.

IMMUNOGEN SYNTHESIS AND ANTISERA TO DELTA-9-THC

For synthetic convenience our initial attempts dealt with the delta-8 isomer of THC. 5′-Carboxy-delta-8-THC was treated with N-hydroxysuccinimide and dicyclohexylcarbodiimide to yield an ester which readily reacted with bovine serum albumin in a mixture of dioxane and sodium bicarbonate solution to yield the desired immunogen containing about 33 residues of delta-8-THC per molecule of bovine serum albumin (4).

This immunogen was injected into rabbits by a modification of the intradermal method of Vaitukaitus et al. (4). Reasonable antiserum titers were achieved by 7.5 weeks after the first immunization and, as we have reported previously, the resulting antisera showed good selectivity for delta-9-tetrahydrocannabinol vs. its metabolites (4,5).

Since the antisera produced from the delta-8-immunogen showed about 2.5 times as great an affinity for delta-8-THC as for delta-9-THC, it appeared that perhaps even better antisera could be achieved starting with a delta-9-5′-carboxylic acid hapten. The desired immunogen was synthesized as in the case of the delta-8-compound. Rabbits were immunized with the resulting conjugate and antisera which were able to bind delta-9-THC were obtained (5). As figure 2 shows, the antisera exhibited very low cross-reactions with the 11-nor-9-carboxy metabolite of delta-9-THC. A significant cross-reaction with the 11-hydroxy metabolite was observed, but this compound is present in very low concentrations in blood after smoking or iv administration (6), so the cross-reaction would be of concern only after oral administration of delta-9-THC.

Analytical Protocol

Vortex mix 100 μ L plasma with 400 μ L methanol

|

Let stand, 5–10 min; centrifuge; aliquot

|

50 μ L aliquot + 400 μ L buffer with radioligand (15,000 cpm)
+ 100 μ L antiserum/buffer

|

Vortex mix; incubate 4–24 hr at 4°C

|

Add charcoal/buffer (500 μ L of 1.58% suspension)

|

Vortex mix; incubate 20 min at 4°C

|

Centrifuge 15 min at 4°C; decant; count.

FIGURE 2. Protocol for analysis of delta-9-THC or its carboxylic acid metabolite in plasma.

RADIOLIGANDS

Now let us turn to consideration of the radioligand. Tritium-labeled radioligands have the advantage of generally better shelf-life. They also have an affinity for the antibody essentially equal to that of the unlabeled compound. On the other hand, iodine-125 has certain advantages over tritium for use as the radioisotope. These include the higher theoretical specific activity, resistance to quenching and ease of measurement. The size of the iodine atom, however, makes its introduction into a small molecule such as THC a problem from the standpoint of binding affinity. Direct substitution of iodine into such a molecule often inhibits binding to the antibody and reduces the affinity below that which can give a useful assay. However, direct introduction of iodine into the 5′-position of THC–that is into the position which was linked to the protein in the immunogen–might allow us to take advantage of the insensitivity of antibody affinity to changes at the position of linkage in the immunogen. Therefore, 5′-iodo-delta-8-THC was prepared and submitted to an exchange reaction with iodine-125 sodium iodide. This procedure led to iodinated THC of moderate specific activity, ranging from about 8-40 Ci/mmole (4-7). More recently we have found that displacement of a 5′-tosylate with iodine-125-iodide can lead to even higher specific activities (7).

ANALYSIS OF DELTA-9-THC IN PLASMA

The above antisera were used to develop an assay for delta-9-THC in plasma. Initially tritium-labeled delta-8-THC of specific activity 60 Ci/mmole (8) was used as the radioligand. Antiserum to delta-9-THC was used at an initial titer of 1:6000. Standard curves showed a practical sensitivity of 25 pg. (We define practical sensitivity as the amount which reduces binding of radioligand by 10%. This is a pragmatic definition, based on experience. Sensitivity limits based on 2 or 3 standard deviations from blank tubes would be much lower.)

For plasma analysis we used the procedure shown in figure 2. Plasma is mixed with methanol (9) and an aliquot of the resulting supernatant is incubated with the radioligand and antiserum, usually overnight, at 4°C. Separation is carried out by addition of a suspension of charcoal in buffer and after centrifugation the supernatant is counted.

Under these conditions our practical sensitivity limit was 2.5 ng/ml with either radioligand. When the tritium radioligand was used, control samples at 8 ng/ml showed a within-assay CV of 5.9% and a between-assay CV of 6.4%. For 30 ng/ml controls these values were 6.0 and 5.5%, respectively. Variances (10) are based on 28 assays and 4 tubes were analyzed per sample. Similar results were seen with the iodinated ligand, but with many fewer analyses.

We have now used both of these ligands in an assay for tetrahydrocannabinol from plasma. Through the courtesy of Dr. John Merritt, University of North Carolina School of Medicine, we obtained some samples from subjects administered delta-9-THC and analyzed them using both ligands. The sensitivity in each case appeared to be 2.5 ng/ml. Figure 3 shows the data from a single subject analyzed using both ligands. Results appear comparable and this conclusion is supported by figure 4 which shows results from the analysis of all 62 samples in which either sample gave results above 2.5 ng/ml. Using tritium as the ordinate, we found a regression equation with a slope of 1.06 and an intercept of 0.72 ng/ml. The correlation coefficient was 0.9907.

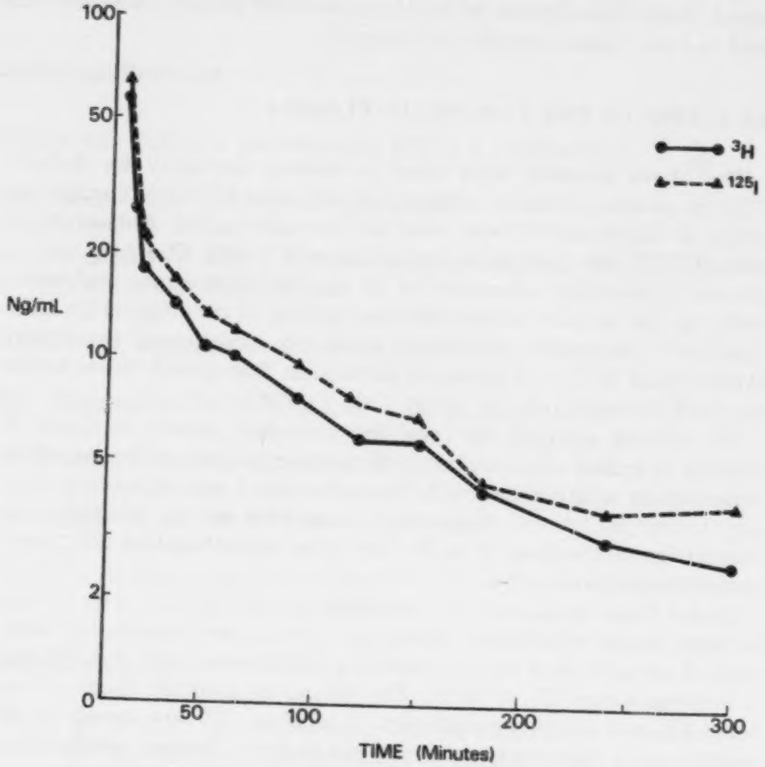


FIGURE 3. Plasma curve from a subject administered delta-9-THC. The methodology of figure 2 and either tritium or iodine-125-substituted radioligands were used.

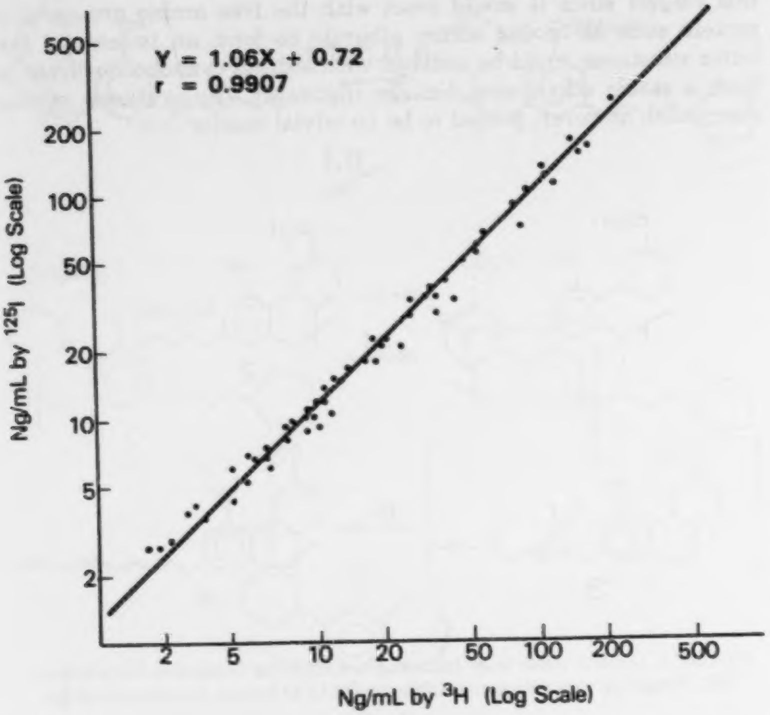


FIGURE 4. Comparison of results on analysis of 62 plasma samples for delta-9-THC with either a tritium or iodine-125-labeled radioligand.

DEVELOPMENT OF SPECIFIC RIA FOR 11-NOR-9-CARBOXY-DELTA-9-THC

Having demonstrated the utility of our approach to the analysis of delta-9-THC, we then set out to apply the same principles to the analysis of various metabolites of delta-9-THC. The 11-nor-9-carboxylic acid (or 11-oic acid) has been shown to be a major metabolite present in plasma and was chosen as our next target for development of an immunoassay. We were reluctant to use a 5′-carboxy analog of this compound as the hapten to prepare an immunogen, since this presented the possibility of obtaining a mixture of 5- and 11-linked conjugates. Therefore it was necessary to find some means of linking through the 5′-position which did not involve a carboxylic acid. An aldehyde moiety would appear to be useful in this respect since it would react with the free amino groups of a protein such as bovine serum albumin to form an imine and the latter substance could be reduced with sodium cyanoborohydride to form a stable alkylamino linkage (figure 5). The synthesis of this compound, however, proved to be no trivial matter (11).

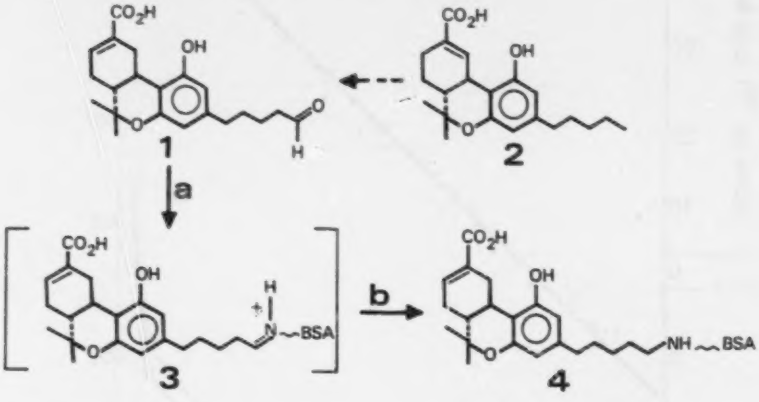


FIGURE 5. General route to an immunogen containing 11-nor-9-carboxy-delta-8-THC. Reagents: a) bovine serum albumin (BSA); b) sodium cyanoborohydride.

Again, for reasons similar to those in the THC series, the delta-8-compound was chosen for synthetic work. In the procedure finally adopted (figure 6), 5′-bromo-delta-8-THC was treated with tetramethyl ammonium acetate to introduce a 5′-acetoxy group. The phenolic group was protected as its t-butyldimethylsilyl ether, and the acetoxy group was selectively removed by treatment with lithium aluminum hydride to give the 5′-alcohol which could be oxidized with pyridinium chlorochromate to the 5′-oxo compound. The aldehyde group was then protected as the ethylene acetal. C-11 was next converted to an aldehyde with selenium dioxide. Oxidation of the aldehyde using sodium cyanide and manganese dioxide in methanol led to the methyl ester of the 9-carboxylic acid. When this ester was saponified with potassium hydroxide and ethanol, both the ester and the silyl protecting groups were cleaved. It then remained only to remove the ethylene acetal to generate the desired compound.

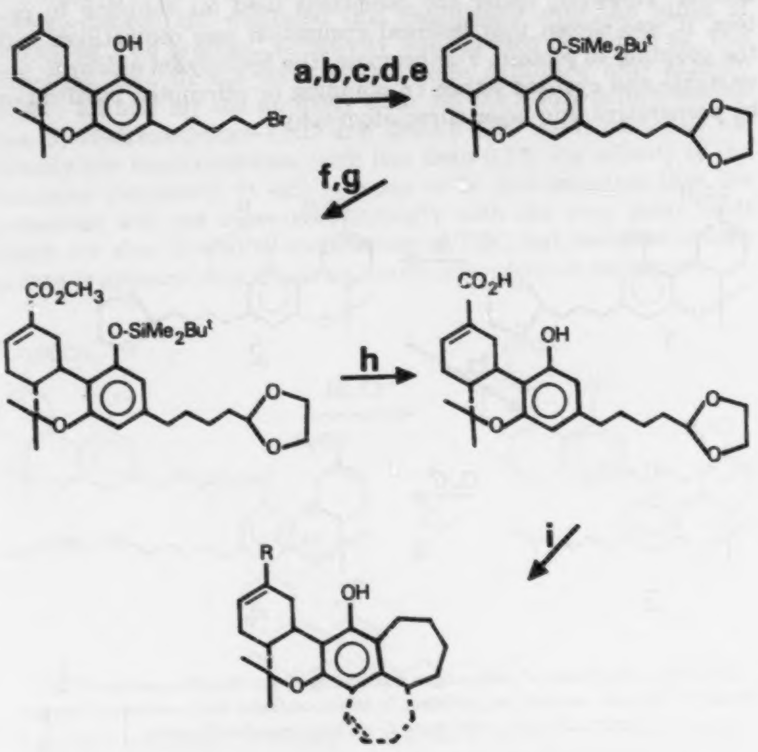


FIGURE 6. Synthesis of ethylene acetal of 5′-oxo-11-nor-9-carboxy-delta-8-THC. Reagents: a) tetramethylammonium acetate; b) t-butyldimethylchlorosilane; c) lithium aluminum hydride; d) pyridinium chlorochromate; e) ethylene glycol, toluenesulfonic acid; f) selenium dioxide; g) sodium cyanide, acetic acid, methanol, manganese dioxide; h) potassium hydroxide; i) toluenesulfonic acid, acetone.

Tests using a model system with a methyl group at C-9 showed that under standard acidic conditions used to hydrolyze acetals, the intermediate aldehyde which formed quickly cyclized by interaction with the reactive phenolic ring. It was therefore necessary to deactivate the phenolic ring by formation of the phenolic acetate (figure 7). This compound could then be hydrolyzed under acidic conditions to give the 5′-oxo compound with the acetate group still present. Mild basic hydrolytic conditions were found to cleave the acetate. However, under the conditions used for coupling to protein, it was shown that internal cyclization was competitive with the coupling to protein. Furthermore, the hydrolyzed aldehyde was unstable and cyclized either on standing or attempted purification by preparative thin-layer chromatography.

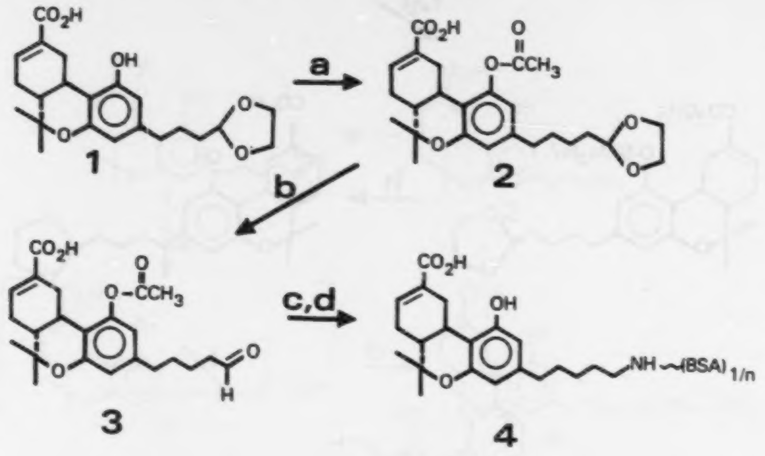


FIGURE 7. Synthesis of immunogen containing 11-nor-9-carboxy-delta-8-THC. Reagents: a) acetic anhydride, pyridine; b) toluenesulfonic acid, acetone; c) bovine serum albumin, methanol; d) sodium cyanoborohydride.

The phenolic acetate therefore was allowed to react with bovine serum albumin in pure anhydrous methanol in the presence of sodium cyanoboro-hydride (12) and the resulting conjugate was deacetylated by stirring with aqueous methanol and sodium bicarbonate. Differential ultraviolet absorption measurements were then used to estimate a minimum incorporation of 11 hapten residues per molecule of protein. Rabbits were immunized with this conjugate. An excellent antiserum titer was obtained by 4 months after initial immunization.

For the radioligand, the methyl ester of 5′-iodo-11-nor-9-carboxy-delta-8-THC was converted to the free acid by treatment with trimethylsilyl iodide followed by hydrolysis with water. Exchange of the resulting 5′-iodocarboxylic acid with iodine-125 sodium iodide led to labeled material (figure 8) with a specific activity of about 7.5 Ci/mmole. Using this iodinated radioligand we then examined the cross-reactions of the anti-serum, which was used at a titer of approximately 1:20,000 initial dilution. As shown in figure 9, 11-nor-9-carboxy-delta-9-THC displaced this radioligand over the range of 20-5000 pg. The practical sensitivity was between 20 and 50 pg. The carboxylic acid in the delta-9-THC series exhibited only slightly greater affinity for the antibody than did the 5′-iodo-delta-8 derivative. 11-Hydroxy-delta-9-THC and delta-9-THC itself exhibited extremely low cross-reactions, with less than 0.1% the affinity of the 9-carboxy compound. It still remains to be demonstrated that the antiserum will not cross-react strongly with the very polar acids which are also formed as metabolites of THC, but based on results to date it appears that this is an extremely selective antiserum.

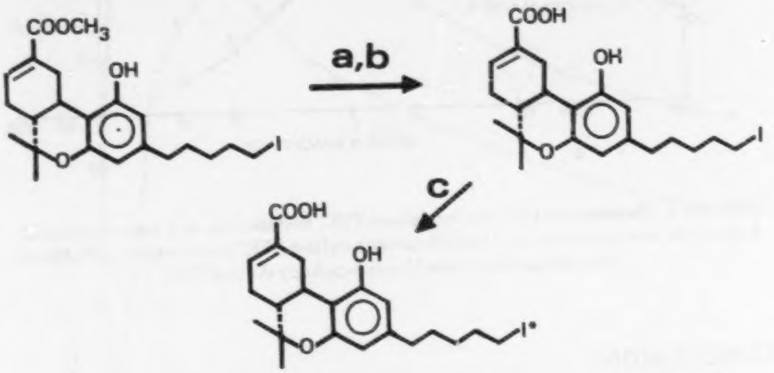


FIGURE 8. Synthesis of radioligand for analysis of 11-nor-9-carboxy-delta-9-THC. Reagents: a) trimethyliodosilane, water; b) iodine-125-NaI, acetone.

ANALYSIS OF 11-NOR-9-CARBOXY-DELTA-9-THC IN PLASMA

We have used this antiserum and radioligand in the analysis of a series of plasma samples provided to us through the courtesy of Mr. Owens and Dr. McBay. Conditions for analysis were the same as for delta-9-THC. Figure 10 shows results for one subject who received THC by smoking. The results of this analysis are in excellent qualitative agreement with data obtained by Wall and coworkers (2). THC itself rises rapidly to a maximum after cessation of smoking and then falls off rapidly. The concentration of the carboxylic acid metabolite rises fairly rapidly, reaching a maximum at around 20-40 minutes and thereafter declining at a slower rate. Almost identical results were observed in a second subject.

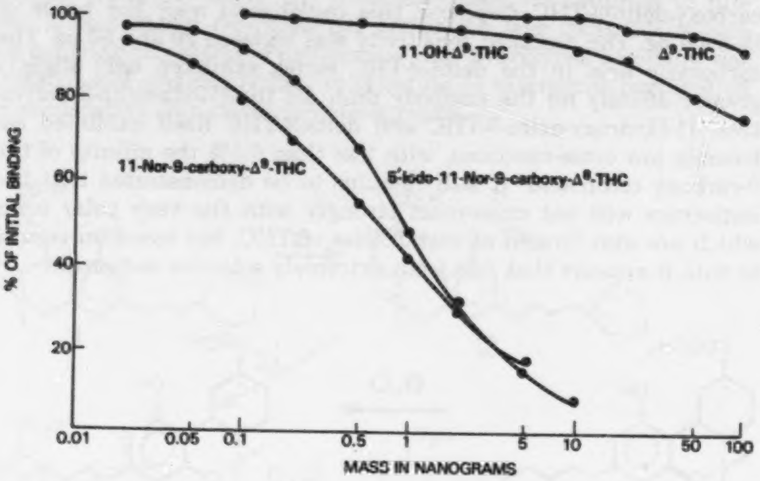


FIGURE 9. Displacement curves for delta-9-THC, metabolites and iodo compounds. Antiserum was generated to 11-nor-9-carboxy-delta-8-THC immunogen; radioligand was iodine-125-5'-iodo-11-nor-9-carboxy-delta-8-THC.

CONCLUSIONS

These studies have demonstrated that linkage of cannabinoid molecules to protein through the 5'-position of the amyl side chain leads to the formation of selective antisera. Antisera developed in this manner can be used for the analysis of plasma samples for both delta-9-THC and 11-nor-9-carboxy-delta-9-THC, a principal plasma metabolite. Furthermore, in conjunction with these antisera, the use of a 5'-iodo radioligand has been shown to be both practical and convenient. As we have previously pointed out, the general concept of introducing the iodine at the point of linkage appears to be one which should have widespread utility in radioimmunoassay work (5). Although the results with the 11-nor-9-carboxy-delta-9-THC analysis are at this point somewhat preliminary and the method requires some further development, it is to our knowledge the first reported example of a highly specific radioimmunoassay for this metabolite in plasma. We are now in the process of applying these general concepts to the preparation of an antiserum for 11-hydroxy-delta-9-THC.

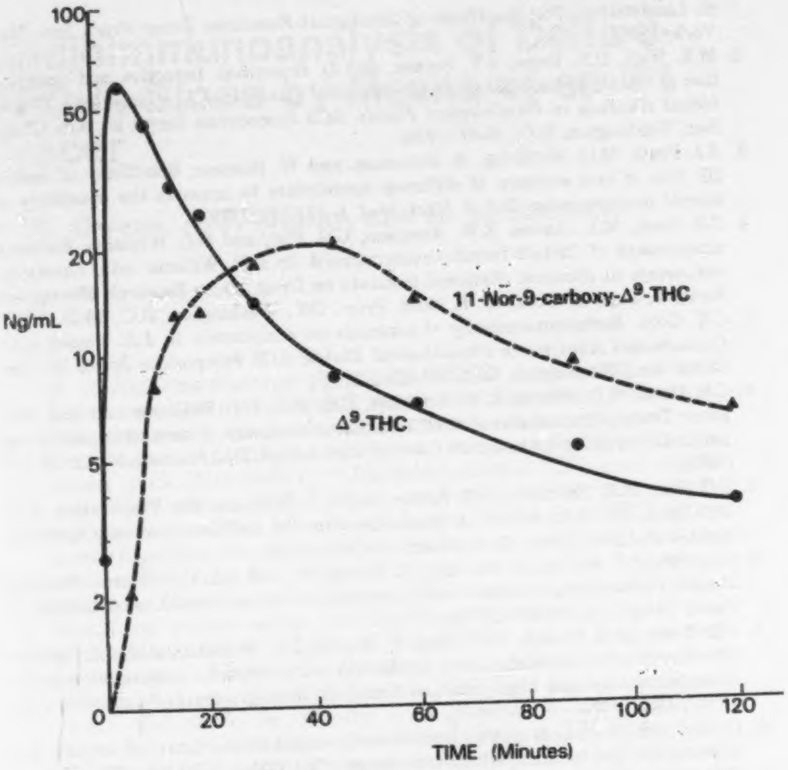


FIGURE 10. Analysis of delta-9-THC and 11-nor-9-carboxy-delta-9-THC in a subject who smoked a single 9 mg THC cigarette.

ACKNOWLEDGMENTS

Dr. Robert Willette originally suggested the synthesis of the 5′-iodo radioligand to us. We also acknowledge helpful discussions with Mr. Mike Owens and Dr. Arthur McBay. Mr. Owens suggested incorporating the methanol extraction procedure into our assay and both Mr. Owens and Dr. McBay have encouraged our development of iodinated radioligands.

REFERENCES

1. K. Landsteiner. *The Specificity of Serological Reactions.* Dover Press, Inc., New York (1962).
2. M.E. Wall, D.R. Brine, J.T. Bursey, and D. Rosenthal. Detection and quantitation of tetrahydrocannabinol in physiological fluids. In J.A. Vinson (ed.), *Cannabinoid Analysis in Physiological Fluids,* ACS Symposium Series 98, Am. Chem. Soc., Washington, D.C., 39-57 (1979).
3. J.J. Pratt, M.G. Woldring, R. Boonman, and W. Bosman. Specificity of assays. III. Use of two antisera of differing specificities to improve the specificity of steroid immunoassay. *Eur. J. Nucl. Med. 4,* 171-177 (1979).
4. C.E. Cook, M.L. Hawes, E.W. Amerson, C.G. Pitt, and D.L. Williams. Radioimmunoassays of Delta-9-Tetrahydrocannabinol. In R.E. Willette (ed.), *Cannabinoid Assays in Humans,* National Institute on Drug Abuse Research Monograph Series 7, Supt. of Docs., U. S. Govt. Print. Off., Washington, D.C., 15-27 (1976).
5. C.E. Cook. Radioimmunoassay of cannabinoid compounds. In J.A. Vinson (ed.), *Cannabinoid Analysis in Physiological Fluids,* ACS Symposium Series 98, Am. Chem. Soc., Washington, D.C., 137-154 (1979).
6. C.E. Cook, M.L. Hawes, E.W. Amerson, C.G. Pitt, D.L. Williams and R.E. Willette. Tetrahydrocannabinol (THC) radioimmunoassay: Immunogen and novel iodine-125-radioligand based on 5′-substituted-delta-8-THC.*Pharmacologist 18,* 291 (1976).
7. C.G. Pitt, H.H. Seltzman, S.R. Setzer, and D.L. Williams. The Preparation of 5′-Iodo-Iodine-125-Delta-8-THC; A Radioligand for the radioimmunoassay of cannabinoids. *J. Label. Comp. Radiopharm., 17,* 681 (1979).
8. C.G. Pitt, P.T. Hobbs, H. Schran, C.E. Twine, Jr., and D.L.M Williams. The synthesis of deuterium, carbon-14 and carrier-free tritium labeled cannabinoids. *J. Label. Comp., 11,* 551-575 (1975).
9. J.D. Teale, J.M. Clough, L.J. King, V. Marks, P.L. Williams, and A.C. Moffat. The incidence of cannabinoids in fatally injured drivers: An investigation by radioimmunoassay and high pressure liquid chromatography.*J. Forens. Sci. Soc., 17,* 177-183 (1977).
10. D. Rodbard. Statistical quality control and routine data processing for radioimmunoassays and immunoradiometric assays. *Clin. Chem., 20,* 1255-1270 (1974).
11. H.H. Seltzman, C.G. Pitt, D.L. Williams, and C.E. Twine. Synthesis of a hapten for the radioimmunoassay of 9-carboxy-11-nor-delta-9-THC. 31st Southeastern regional meeting of the American chemical society, Abstract No. 249 (1979).
12. Cf.R. Muller, A. Scheuer, H. Gerdes and K.-O Mosbach. Direkte kupplung von steroiden an einweiss durch reduktive aminierung zur gewinnung spezifischer antikorper. *Fresenius Z. Anal. Chem., 290,* 164 (1978).

AUTHORS

C. E. Cook, H. H. Seltzman, V. H. Schindler,
C. R. Tallent, K. M. Chin, and C. G. Pitt
Research Triangle Institute
Research Triangle Park, N. C. 27709

Analysis of Cannabinoids
Research Monograph 42, R. Hawks, Editor
National Institute on Drug Abuse, 1982

Radioimmunoanalysis of Delta-9-THC in Blood by Means of an ^{125}I Tracer

S. M. Owens, A. J. McBay, and H. M. Reisner

A radioimmunoassay for delta-9-THC in plasma, whole blood, or hemolyzed blood specimens has been presented. Samples and standards were diluted with methanol and centrifuged. An aliquot of the supernatant fluid was incubated with RIA buffer, ^{125}I-labeled delta-8-THC and rabbit anti-THC serum. Solid phase goat anti-rabbit immunoglobulins were added to separate bound from free THC. After centrifugation the supernatant fluid was aspirated and the radioactivity of the precipitate was counted in a gamma counter. The concentration of THC was calculated from a standard curve using the logit-log transformation of the average counts of duplicate tubes. The assay had several advantages. Methanol dilution gave better results than direct analysis. The ^{125}I-labeled THC had high specific activity and could be counted in a gamma counter. The immunological separation of antibody-bound THC from free THC was better than separation techniques using ammonium sulfate and activated charcoal. THC was determined in 0.1 ml of sample with a sensitivity of 1.5 ng/ml in plasma and 3.0 ng/ml in hemolyzed blood.

INTRODUCTION

There is a need for accurate, sensitive and specific radioimmunoassays (RIA) of delta-9-THC (THC) for pharmacological and forensic studies of various biological fluids. The RIA of plasma samples (1,2) and hemolyzed blood (3) have been reported.

Forensic blood samples are usually grossly hemolyzed. These require a RIA that is not affected by varying degrees of hemolysis.

An ^{125}I-labeled THC radioligand is needed for this application (4). The advantages of ^{125}I-THC over ^{3}H- or ^{14}C-labeled THC are that it is not affected by color quenching during the counting of radioactivity and it can be synthesized at a higher specific activity (5).

THC is a highly lipophilic molecule that adheres to glass and plastics and binds to various blood products. We found that a critical step in the RIA of THC was the separation of antibody-bound radioactivity from other radioactivity. We compared five common RIA separation methods and found that a solid phase second antibody improved the separation.

PREPARATION OF ANTI-THC SERA AND RADIOLIGAND

Delta-8-THC was conjugated to bovine serum albumin by the method of Cook et al (1). Delta-8-THC was used to make the antigen because delta-9-THC was not available. The concentration of delta-8-THC in marijuana, if present, is very low compared to the concentration of delta-9-THC. It therefore will not contribute to the concentration of delta-9-THC in this analysis.

Four New Zealand white rabbits were each injected with 200 μg of the antigen in physiological saline emulsified with Freund's complete adjuvant. All injections were administered subcutaneously near regional lymph centers. The next four boosts were given every 3 weeks with 100 μg of antigen plus Freund's complete adjuvant. Seven months from the start of immunization the rabbits were boosted with 100 μg of antigen and Freund's incomplete adjuvant. The serum was tested for THC binding after the fourth boost and on all subsequent boosts. The titer was expressed as that dilution of serum which bound 50% of 85 pg of the radioligand. The assay conditions were those stated in the procedure except that ammonium sulfate was used to separate bound from free radioactivity. The serum from the rabbit with the highest titer was chosen for use in the assay.

The radioligand 5′-iodo-^{125}I-delta-8-THC was synthesized in our laboratory by the method of Pitt et al (5). The specific activity was determined to be 250 Ci/mmole by the self-displacement method of Morris (6).

SPECIFICITY OF ANTISERUM

The cross-reactivity of the antiserum was measured by determining the concentration of compound that caused 50% displacement of the radioligand. Since delta-9-THC was the analyte of interest, its concentration at 50% displacement was considered as 100% cross-reactivity. All other compounds were compared to it (table 1). The assay conditions were those described in the procedure.

Compound	%
Delta-9-THC	100
Delta-8-THC	420
11-OH-delta-9-THC	18
Cannabinol	4
Cannabidiol	< 0.6
11-nor-delta-9-THC-9-COOH	< 0.1
Diazepam	< 0.01
Secobarbital	< 0.01
d-Amphetamine	< 0.01
Morphine Sulfate	< 0.01
Amitriptyline	< 0.01
Phencyclidine	< 0.01
Testosterone	< 0.001
Hydrocortisone	< 0.001
Progesterone	< 0.001

TABLE 1. Cross reactivity of various cannabinoids, drugs, and steroids with anti-delta-8-THC serum. All percent cross reactivities preceded by the symbol for "less than" gave no indication of cross reactivity at the concentration equivalent to that percentage.

SAMPLE PREPARATION

THC in absolute ethanol was obtained from the Research Triangle Institute (RTI), Research Triangle Park, NC. Standards and controls were prepared the day of the analysis by adding THC in absolute ethanol to plasma or hemolyzed blood sample pools. Standards were prepared from 1-100 ng/ml. Hemolyzed blood was made by storing whole blood for at least one month at 4°C with 1% sodium fluoride as a preservative. All standards, controls, and unknowns were treated the same throughout the procedure.

Sensitivity was increased and interferences were decreased by diluting the samples with methanol rather than analyzing the samples directly. The use of methanol has been reported by Teale et al. (7).

An aliquot (100 μl) of sample was pipetted into a silanized 10 × 75 mm glass test tube. The tube was vortexed at low speed while methanol (500 μl) was added. The tubes were immediately corked. After 30 minutes the tubes were centrifuged at 1000 × g for 15 minutes. A 100 μl aliquot of the supernatant fluid was used in the assay. When approximately 10 ng/ml of the radioligand was added to plasma or hemolyzed blood (n=10) the percent recovery was 99.3 (%CV=1.4) and 93.3 (%CV=4.7) respectively.

RADIOIMMUNOASSAY PROCEDURE

The buffer was tris-NaCl with bovine serum albumin (0.05 M tris(hydroxymethyl)-aminomethane and 0.15 M NaCl with 0.1% bovine serum albumin and 0.2% NaN_3 adjusted to pH 7.6 with concentrated HCl). The radioligand in 50% ethanol was added to the buffer in a ratio of 40 pg per 500 μl. A 500 μl aliquot of radioligand in buffer was added to untreated 10 × 75 mm glass test tubes. Each sample was analyzed in duplicate. An aliquot (100 μl) of the methanol supernatant fluid was added to appropriate tubes and vortexed. The anti-THC serum was diluted 1:35,000 with buffer (20-35% antibody specific binding) and 100 μl was added to all tubes except control tubes for nonspecific binding and total counts. To those tubes 100 μl of buffer was added. All tubes were vortexed. The rack of tubes was sealed in a plastic bag to prevent evaporation and was incubated overnight at 4°C. The next day, 100 μl of solid phase goat anti-rabbit immunoglobulins (Immunobeads, prepared by Bio-Rad Laboratories, Richmond, CA 94804) were added. The tubes were vortexed and then refrigerated at 4°C for 1 hour. The tubes were allowed to warm to room temperature for 1 hour. They were then centrifuged at 1000 × g for 8 minutes to precipitate antibody-bound radioligand. The supernatant was aspirated without disturbing the pellet and 2 ml of buffer were added. The tubes were vortexed and centrifuged as before. The supernatant fluid was aspirated without disturbing the pellet. This washing reduced specific and nonspecific binding approximately 5%. The tubes were counted in a gamma counter, having an 84.6% counting efficiency with a ^{125}I source. The concentration of THC in controls and unknowns was obtained from a standard curve using the logit-log transformation of the average counts of duplicate tubes.

COMPARISON OF SEPARATION METHODS

The methods commonly used to separate free from antibody-bound radioactivity are based on chemical or immunological differences between free and antibody-bound radioactivity. If the equilibrium between the bound and free forms is disturbed a misclassification of counts could occur. An ideal method should not affect this equilibrium. Five radioimmunoassay separation methods were compared. These used polyethylene glycol, Staphylococcus aureus with protein A, activated charcoal, ammonium sulfate, or solid phase goat anti-rabbit immunoglobulins (second antibody). The factors considered in choosing a method were the accuracy of classification of bound and free counts and the variability of binding with plasma and hemolyzed blood. The Staphylococcus aureus with protein A and polyethylene glycol methods were not usable due to high nonspecific binding and variability from sample to sample. Ammonium sulfate performed well with plasma even though there was high nonspecific binding. It could not be used with hemolyzed blood. The charcoal separation performed well with plasma. With hemolyzed blood it had variable nonspecific binding with different samples. Also, a refrigerated centrifuge was needed. The solid phase second antibody separation gave the most consistent results with different specimens. The nonspecific binding for plasma was consistently 0.5% ± 0.1% and for hemolyzed blood it was 1.5% ± 0.2%. Another important feature was that standard curves of plasma and hemolyzed blood, when assayed in the same RIA, were almost superimposable (see figure 1). Since plasma had 2.8% greater antibody-specific binding of radioligand than hemolyzed blood, all samples had to be compared to their own zero dose standard, i.e., plasma unknowns to plasma zero dose standards and hemolyzed blood unknowns to hemolyzed blood zero dose standards.

VALIDATION OF PROCEDURE

To obtain blood containing THC, a smoking study was conducted under the direction of Dr. Mario Perez-Reyes of the University of North Carolina Department of Psychiatry. Mexican marijuana cigarettes containing 1% delta-9-THC (approximately 9 mg) and other naturally occurring cannabinoids were provided by the National Institute on Drug Abuse. Each experienced male and female volunteer smoked on two separate occasions either a marijuana cigarette or a placebo. The placebos, which were obtained from NIDA, were prepared by extracting cannabinoids from marijuana.

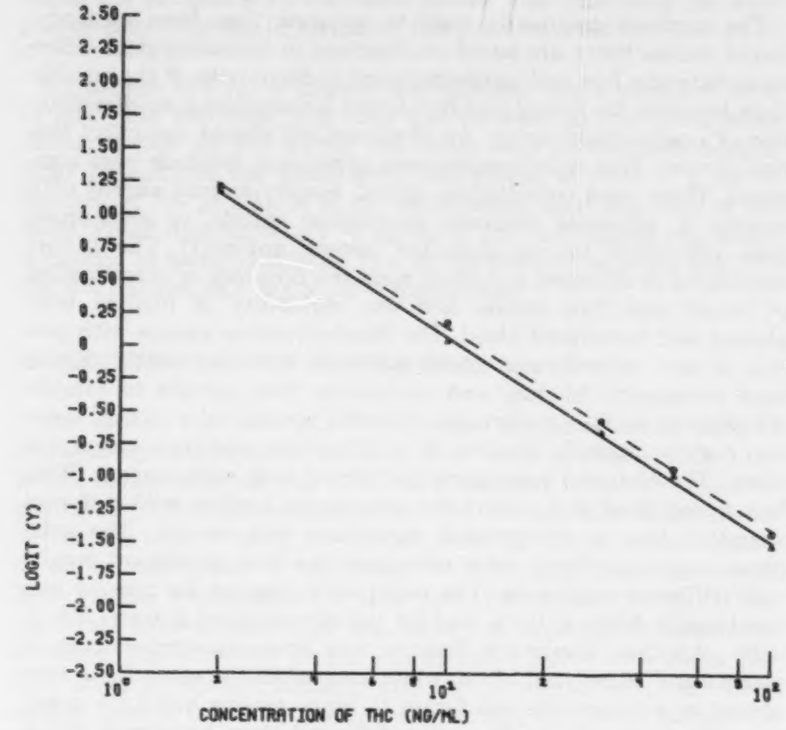


FIGURE 1. Typical RIA logit-log curves for plasma and hemolyzed blood. When assayed in the same RIA they were almost superimposable. The plasma and hemolyzed blood curves had slopes of -1.56 and -1.55, y-intercepts of 1.63 and 1.70, and nonspecific bindings of 0.5% and 1.5%, respectively. Both curves had a r2=0.999. The percentage of total binding was 2.8% less for hemolyzed blood than for plasma.

All subjects were asked not to use marijuana for 4 days prior to the study. Blood samples were collected from an antecubital vein at 0, 5, 10, 15, 20, 30, 45, 60, 90, and 120 minutes after the start of smoking. A portion of the whole blood was preserved with 1% sodium fluoride. The remaining blood was heparinized and the plasma separated. All samples were stored in silanized scintillation vials at −20°C until analyzed.

A total of 50 positive plasma samples from 5 subjects were analyzed in a double-blind fashion. The results were compared with time after smoking. This was to determine if the expected concentration vs. time curve would be produced (see figure 2). The biphasic curve that was produced was similar to that of Wall et al (8).

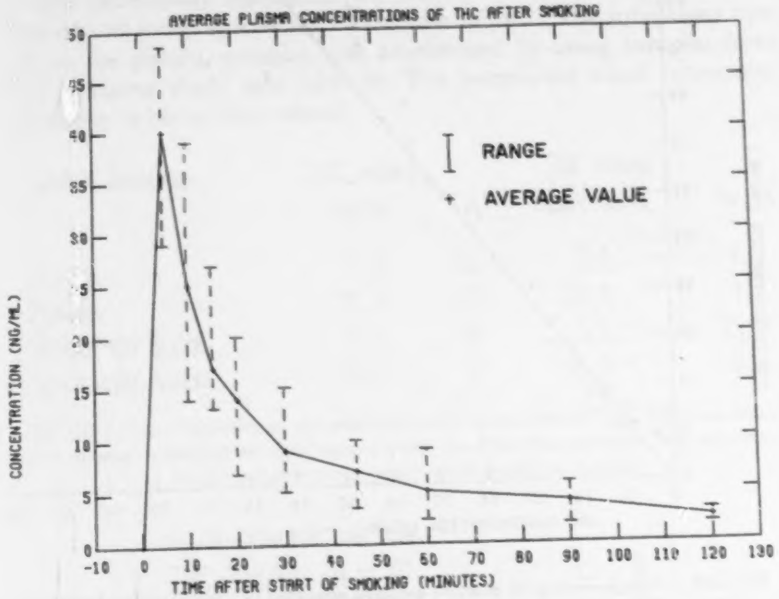


FIGURE 2. Five subjects smoked Mexican marijuana cigarettes that contained approximately 9 mg of THC. It took from 7.5 to 13 minutes to finish the cigarettes. The concentration vs. time curves were all biphasic and similar for each subject.

As further validation, 31 positive and 21 negative plasma samples from the smoking study were analyzed in double-blind fashion at RTI by the delta-9-THC RIA of Dr. C. E. Cook and by our procedure (see figure 3). The correlation coefficient was 0.971. All negative samples were correctly identified by both laboratories.

The usual means of determining sensitivity of a RIA is to evaluate statistically the counting variances of zero dose standards (9). By this criterion, the sensitivity for THC in plasma and hemolyzed

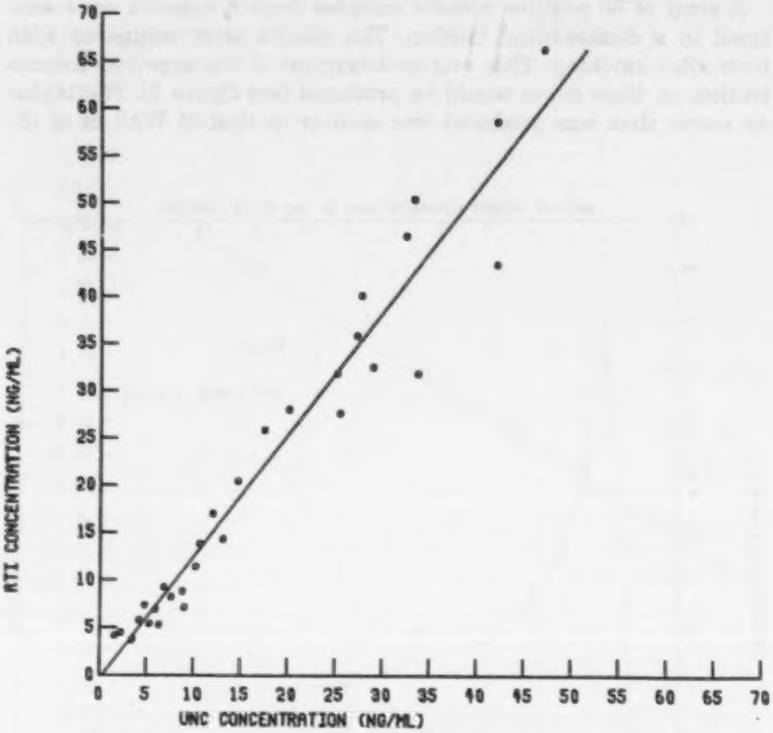


FIGURE 3. Comparison of 31 positive samples analyzed in a double-blind fashion at RTI by a THC RIA and by our THC RIA. The correlation coefficient was 0.971.

blood specimens would be less than 1 ng/ml. The analysis of presumed negative samples proved to be a more rigid criterion for sensitivity estimation.

The sensitivity was determined by comparing the calculated concentrations of all negative samples with the zero dose standards. Negative blood samples were obtained from subjects smoking placebos and from 10 nonusers of marijuana. Altogether 61 negative plasmas and 16 negative hemolyzed bloods from 16 different subjects were analyzed. No plasma was calculated greater than 1.0 ng/ml and no hemolyzed blood was calculated greater than 2.5 ng/ml. To assure no false positives, the least detectable concentration was arbitrarily set at 1.5 ng/ml in plasma and 3.0 ng/ml in hemolyzed blood.

We are presently trying to confirm if all samples from the placebo smoking study are THC-free. Some had significantly higher concentrations than the concentrations found in nonusers of marijuana. Since individuals in the smoking study were marijuana users, the low concentrations of THC found in some samples may represent low residual concentrations of the drug.

The intra-assay reproducibility was determined using plasma or hemolyzed blood spiked with THC (see table 2). The interassay precision for plasma samples was determined by using samples from the smoking study (see table 3). The hemolyzed blood interassay precision is being determined.

SAMPLE ASSAYED	THC ADDED, NG/ML	N	THC FOUND MEAN NG/ML	CV, %
PLASMA	5.0	10	5.1	7.9
PLASMA	30.0	10	27.9	4.2
HEMOLYZED BLOOD	5.0	8	5.5	15.0
HEMOLYZED BLOOD	30.0	8	31.3	5.9

TABLE 2. Reproducibility of the assay of known quantities of THC. THC was added to a plasma or hemolyzed blood sample pool. The THC concentration was calculated from standard curves which were similar to figure 1.

MEAN CONCENTRATION THC, NG/ML (N = 8)	CV, %
33.1	7.7
10.7	11.0

TABLE 3. The interassay precision was determined using plasma samples from a marijuana smoking study. The replicates were analyzed in separate assays over a 3-week period.

CONCLUSIONS

The concentration of THC after smoking can be accurately determined in various biological fluids by this RIA, using an ^{125}I tracer and a specific separation method. This assay will be of use in forensic and pharmacological studies.

ACKNOWLEDGMENTS

Supported in part by North Carolina Governor's Highway Safety Program Project No. 80-08-03-C-308-2.

The helpful discussions with Dr. C. E. Cook and the technical advice during preparation of the radioligand by Dr. C. G. Pitt, D. L. Williams, and Dr. H. H. Seltzman of the Research Triangle Institute are acknowledged. Dr. Mario Perez-Reyes of the University of North Carolina at Chapel Hill performed the human studies.

REFERENCES

1. C.E. Cook. M.L. Hawes, E.W. Amerson, C.G. Pitt, and D.W. Williams. Radioimmunoassays of delta-9-tetrahydrocannabinol. In R.E. Willette (ed.), *Cannabinoid Assays in Humans*, National Institute on Drug Abuse Research Monograph 7, Supt. of Docs., U.S. Govt. Print. Off., Washington, D.C., 15-27 (1976).
2. S.R. Gross and J.R. Soares. Validated direct blood delta-9-THC radioimmune quantitation. *J. Anal. Toxicol.*, *2*, 98-100 (1978).
3. S.R. Gross, J.R. Soares, and V.C. Reeve. (Personal communication).
4. C.E. Cook. Radioimmunoassay of cannabinoid compounds. In J.A. Vinson (ed.), *Cannabinoid Analysis in Physiological Fluids*, ACS Symposium Series 98, Am. Chem. Soc., Washington, D.C., 137-154 (1979).
5. C.G. Pitt, H.H. Seltzman, S.R. Setzer, and D.L. Williams. The preparation of 5'-iodo-^{125}I-delta-8-THC; a radioligand for the radioimmunoassay of cannabinoids. *J. of Labeled Compd and Radiopharm*, *17*, 681-689 (1980).
6. B.J. Morris. Specific radioactivity of radioimmunoassay tracer determined by self-displacement: A re-evaluation. *Clin. Chim. Acta*, *73*, 213-216 (1976).
7. J.D. Teale, J.M. Clough, L.J. King, V. Marks, P.L. Williams, and A.C. Moffat. The incidence of cannabinoids in fatally injured drivers: An investigation by radioimmunoassay and high pressure liquid chromatography. *J. Forens. Sci. Soc.*, *17*, 177-183 (1977).
8. M.E. Wall, T.M. Harvey, J.T. Bursey, D.R. Brine, and D. Rosenthal. Analytical methods for the determination of cannabinoids in biological media. In R.E. Willette (ed.), *Cannabinoid Assays in Humans*, National Institute on Drug Abuse Research Monograph 7, Supt. of Docs., U.S. Govt. Print. Off., Washington, D.C., 107-117 (1976).
9. D. Rodbard. Statistical estimation of the minimal detectable concentration ("sensitivity") for radioligand assays. *Anal. Biochem.*, *90*, 1-12 (1978).

AUTHORS

S. M. Owens* and A. J. McBay
Office of the Chief Medical Examiner
and
Department of Pathology
University of North Carolina
Chapel Hill, N. C. 27514
and
H. M. Reisner
Department of Pathology
University of North Carolina
Chapel Hill, N. C. 27514

*present address:
Department of Pharmacology
University of Arizona
Tucson, Arizona

Analysis of Cannabinoids
Research Monograph 42, R. Hawks, Editor
National Institute on Drug Abuse, 1982

Significant Developments in Radioimmune Methods Applied to Δ^9-THC and its 9-Substituted Metabolites

J. R. Soares, J. D. Grant, and S. J. Gross

Novel RIA's have been developed for marijuana detection. Modified simple techniques measure the "acute" metabolites in minute saliva, breath, and dried blood samples. Solid purified antibody matches the sensitivity and yields sensitivity values identical to liquid assays. The importance of the polar 9-substituted metabolites (9SM) of THC in acute users is shown by time course data that indicate their levels remain high in the critical post-intoxication period when Δ^9-THC clears from the blood. Therefore a single-tube assay using solid-phase reagents was constructed to quantitate simultaneously Δ^9-THC and 9SM. When Δ^9-THC values are low or questionable in subject samples, 9SM would appear useful to confirm or refute acute marijuana use.

INTRODUCTION

The evolution of radioimmunoassay (RIA) techniques has confirmed our original concept (1) that desirable specificity depends on sterically free chemical functions in the hapten and marker moieties.

These principles have allowed the development of Δ^9-tetrahydrocannabinol (THC) and polar 9-substituted THC metabolite (9SM) assays requiring a single incubation of 20 µl samples of serum or blood (hemolyzed). These sensitive assays (5 ng/ml) have routine clinical applicability. For example, THC dosage for antiemetic effects can effectively be monitored by serum concentration.

Furthermore, such determinations will add substantially to clinical epidemiologic and behavioral assessments. Given a high avidity antibody, the assay is flexible and can encompass a broad spectrum of liquids and solids. Since there are obvious forensic applications, the existing methodology has been extended to breath.

Conventional RIA procedures require select reagents and critical steps mainly to separate free radioligand from that bound to antibody. This can be accomplished by "pour out" or "dipstick" techniques utilizing newly developed solid matrices containing covalently bound antibody or hapten. These materials can now be used to measure two (or more) different entities simultaneously in a single container.

MATERIAL AND METHODS

Subjects

Fifteen adult male volunteers were recruited. Their marijuana habits ranged from light (<1/month) to heavy (>3/day). They were asked to refrain from private use of cannabis for 24 hours prior to testing and the baseline (pre-smoke) negativity of their samples (blood and breath) verified by RIA. Volunteers were allowed 15 minutes to complete a single marijuana cigarette containing THC equivalent to approximately 200 μg Δ^9-THC per kg body weight of subject. Blood and breath were collected at various intervals up to 6 hours after smoking. Serum was obtained by centrifugation of samples in red top vacutainer tubes. Five uninterrupted breaths were passed over a borosilicate solid matrix. Cannabinoids were recovered by elution with ethanol, reconstituted in buffer and tested for Δ^9-THC and 9SM content by liquid phase methods (described below).

Haptens and Antigens

The 2-(4′-carboxyphenylazo) derivatives of Δ^9-THC (Azo-THC), 11-hydroxy-Δ^9-THC (Azo-11-OH) and cannabinol (Azo-CBN) were covalently coupled to polymers for immunization (2) and affinity chromatography (3).

Antisera

Texas bush goats were immunized with Keyhole Limpet Hemocyanin conjugates of Azo-THC and Azo-11-OH as previously described (2). Sera harvested by plasmapheresis were tested as follows.

Titer was defined as the dilution at which 0.1 ml of antiserum binds 40% of added ^{3}H-Δ^{9}-THC (40,000 cpm). Cross reactivities were calculated from standard curves and expressed as Δ^{9}-THC/cannabinoid mass ratio at 50% inhibition (× 100%).

Diluted THC-specific antiserum was used directly in standard liquid-phase assay. 9SM antibodies isolated by affinity chromatography were coupled to an insoluble matrix for solid-phase methods.

Immunosorbant

The Azo-CBN carboxyl (50 μmoles) was converted to an active ester by isobutyl chloroformate (60 μmoles) and added to a 10% suspension of aminoalkyl agarose (Affi-Gel 102, BioRad, 35.1 μmoles of alkylamine) in dioxane. The immunosorbant (Azo-CBN-S) was washed with dry dioxane (1L) to remove uncoupled hapten, and stored in distilled water.

Affinity Chromatography

Saturated ammonium sulfate was added to Azo-11-OH antisera to a final concentration of 33% and the precipitate (crude IgG) further purified by passage through QAE Sephadex (QAE IgG). Antibody dilutions were concentrated (10 ng/ml) and 10 ml aliquots were incubated with immunosorbant to remove all antibody from the supernate. Azo-CBN-S was washed with PBS (0.01 M phosphate buffer, pH 6.9, 0.9% sodium chloride) until free of nonspecific protein (O.D. < .005). There was a final wash with 1 M lithium bromide (100 ml). Clean immunosorbant was pipetted into glass columns (1.2 × 15 cm). Elution with 0.09 M hydrochloric acid (30 ml) removed specific antibody. The eluate was dialyzed against distilled water (12 hours at 4°C) and phosphate buffer (24 hours at 4°C).

Purified antibody was concentrated with sucrose and concentrations calculated spectrophotometrically (280 nm). Final titer was compared with parent solutions to document recovery of activity.

Matrix Antibody Conjugates

Antibody fractions of varying purity were coupled to a polyfunctional acrylate. Active polymer fractions (33 mg) were reacted with 400 μg per ml antibody (3.0 ml). Uncoupled IgG was removed with PBS. Binding efficiency of immunosorbant-purified antibody-matrix conjugate (Ab-C) was compared with IgG-polymer controls (nonimmune IgG, crude IgG and QAE IgG fraction). Solid-phase reagents (30% suspension in PBS) were stored at 4°C.

Liquid-Phase Radioimmunoassays

The following cannabinoids were obtained from the National Institute on Drug Abuse (NIDA): Δ^9-THC, Δ^8-THC, 11-hydroxy-Δ^9-THC, 11-nor-Δ^9-THC-9-carboxylic acid, cannabinol, cannabidiol, and ^{3}H-Δ^9-THC (50.3 Ci/mM).

Standard inhibition curves were constructed from non-user samples containing known amounts of Δ^9-THC. Serum (50 μl) was incubated (16 hours, 4°C) with 0.1 ml antiserum (diluted to bind 40% of added marker in the absence of unlabeled THC) and 188 pg (40,000 cpm) of ^{3}H-Δ^9-THC. Free and antibody-bound radioligands were separated using 0.5 ml dextran-coated charcoal suspension (50 gm Norit A charcoal and 5 gm dextran T-70 in 1.0 L PBS) chilled at 4°C by refrigeration. Antibody-bound ^{3}H-Δ^9-THC was counted in a Beckman LS-250 Beta counter after decanting supernates into scintillation vials containing 10 ml of Aquasol (New England Nuclear).

Logit transformation (4) was employed to construct standard curves and to calculate Δ^9-THC concentrations of unknown samples using a Wang 2200 computer.

Solid-Phase 9SM RIA

Serum standards were prepared from pooled marijuana negative serum. Stock solutions of 11-nor-Δ^9-THC-9-carboxylic acid in 50% ethanol (0.01 mg/ml) were diluted 1/100 with serum to give a final concentration of 100 ng/ml. This was serially diluted in serum for standard curves which were included in each assay by transferring aliquots (20 μl) from a sequence of cannabinoid concentrations into 10 × 75 mm borosilicate glass tubes in duplicate. Unknown samples (20 μl) were assayed identically.

Ab-C (10 μl) and ^{3}H-Δ^9-THC (30,000 cpm/0.6 ml PBS, 0.1% albumin) were added and the tubes incubated for 12 hours at 4°C with intermittent shaking. PBS (1.0 ml) was added, and the tubes vortexed. After centrifugation (4,000 rpm, 10 minutes), supernates were discarded, and the percentage of ^{3}H-Δ^9-THC bound to antibody calculated by counting the matrix directly in scintillation fluid. Log-logit standard curves were used to estimate unknown 9SM concentrations.

Dual-Metabolite RIA

The foregoing method was modified to assay Δ^9-THC and 9SM simultaneously in a single tube. Δ^9-THC and 9SM serum standards were added to separate sets of 10 × 75 mm borosilicate glass tubes in duplicate. ^{3}H-Δ^9-THC (30,000 cpm/0.1 ml), Ab-C (10 μl) and soluble Δ^9-THC specific antibody (0.5 ml) were added to each set and the reagents incubated (12 hours, 4°C) with intermittent shaking. The tubes were centrifuged and the percent of label bound to 9SM antibody determined by measuring matrix counts. ^{3}H-Δ^9-THC bound to Δ^9-THC specific antibody was measured in the supernate. Unknown samples (20 μl) were assayed identically, and Δ^9-THC and 9SM concentrations estimated from liquid (Δ^9-THC) and solid (9SM) phase standard curves.

RESULTS

Antisera Specificities

Cross-reactivities are summarized in table 1. 11-Hydroxy-Δ^9-THC, 11-nor-Δ^9-THC-9-carboxylic acid and Δ^9-THC are equally recognized by the 9SM antibody. The non-homologous radioligand (^{3}H-Δ^8-THC) contributed to this spectrum. Δ^9-THC antiserum has far greater specificity, the cross-reactivities for 11-hydroxy-Δ^9-THC and 11-nor-Δ^9-THC-9-carboxylic acid being 26% and 0.5% respectively.

TABLE 1. Percent Crossreactivities*

	Antiserum	
Compound	9SM	Δ^9-THC
Δ^9-THC	100.0	100.0
Δ^8-THC	98.2	98.5
11-hydroxy-Δ^9-THC	97.6	26.0
11-nor-Δ^9-THC-9-carboxylic acid	96.5	0.5
Cannabinol	15.5	22.0
Cannabidiol	0.1	0.15

*Δ^9-THC/cannabinoid ratio at 50% inhibition (x100%).

Cannabinoid Blood Levels

Pre-intoxication levels of 9SM and Δ^9-THC were negative in sera from all 15 volunteers. Initial samples taken 15 minutes after completion of smoking showed Δ^9-THC concentrations between 9 and 42 ng/ml. Thereafter, levels rapidly decreased, becoming undetectable at 2 1/4 to 3 1/4 hours. 9SM levels were vastly higher (20-198 ng/ml) than corresponding Δ^9-THC values. The persistence of metabolites was longer than Δ^9-THC in all subjects but varied with their individual habits. For example, in all three subjects who used marijuana heavily (>3 times/day) 9SM levels remained elevated (>20 ng/ml) up to 6 hours after marijuana inhalation (figure 1). 9SM returned to baseline (0) in two subjects whose sera were obtained at 24 hours. Alternatively, 9SM decreased to the level of assay sensitivity (5 ng/ml) in all moderate smokers (1-4 times/week) by 5 1/2 hours. Finally, two light smokers' (<1 time/month) sera contained no measurable 9SM 3 1/4 hours following acute exposure.

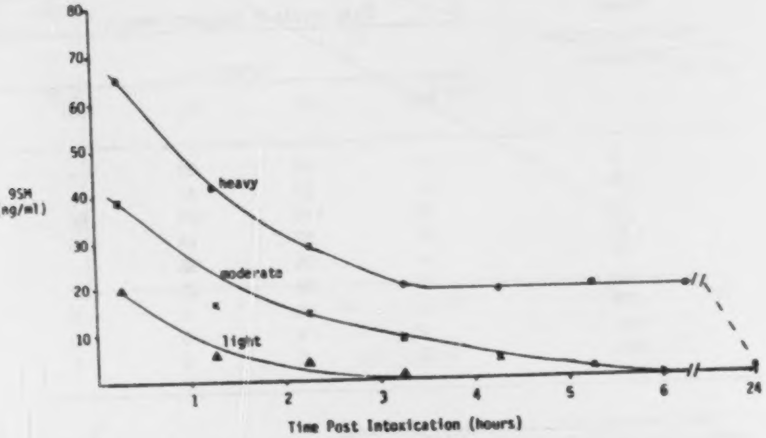


FIGURE 1. Serum levels of Δ^9-THC 9-polar metabolite in individuals who differ in degree of marijuana use. Levels of 9SM were determined at various intervals after subjects smoked one marijuana cigarette containing 200 μg Δ^9-THC/Kg body weight.

In all cases, 9SM cleared more slowly from the blood than Δ^9-THC. Indeed, the mean 9SM/Δ^9-THC ratio for these subjects increased from 2.9 at 15 minutes to 5.7 at 2 1/2 hours.

Breath

Cannabinoids were measured in trapped breath from ten of the above volunteers after smoking a single marijuana cigarette. Both Δ^9-THC and 9SM were detected immediately upon completion of smoking (0 time). Levels of Δ^9-THC between 10 and 56 ng/sample were measured at 15 minutes after smoking and were detectable to

1 hour. Higher concentrations of 9SM were noted (table 2) with maximum values (50-123 ng/sample) observed at 15 minutes. Significant amounts of 9SM were detected up to 2 1/2 hours.

TABLE 2. Δ^9-THC Metabolites in Breath at Various Intervals after Smoking a Single Marijuana Cigarette (200 μg Δ^9-THC/Kg of body weight)

Time* Post-intoxication (minutes)	9SM levels in trapped breath**			
	Subjects			
	RR	EP	JT	DG
0	—	21	31	—
5	—	27	47	—
15	62	110	123	50
30	58	57	19	30
45	—	35	38	—
60	26	28	23	24
120	15	8	8	6
150	2	4	0	0
180	0	0	0	0

*Zero time signifies completion of smoke.

**Trapped breath was analyzed by liquid-phase methods using spiked-buffer standards and 9SM level expressed as ng recovered per sample.

Solid-Phase 9SM Assay

Standard Curves

Ab-C and QAE IgG-C standard curves are compared in figure 2. Six replicate assays were used to construct the Ab-C curve. Log-logit transformation of this data yielded a linear response to 9SM in the 2-50 ng/ml range. The uniformly low coefficients of variation for each standard documented precision throughout the response curve. Assay sensitivity (2 ng/ml) was defined as the point on the standard curve greater by 2 standard deviations than 0.0 ng/ml.

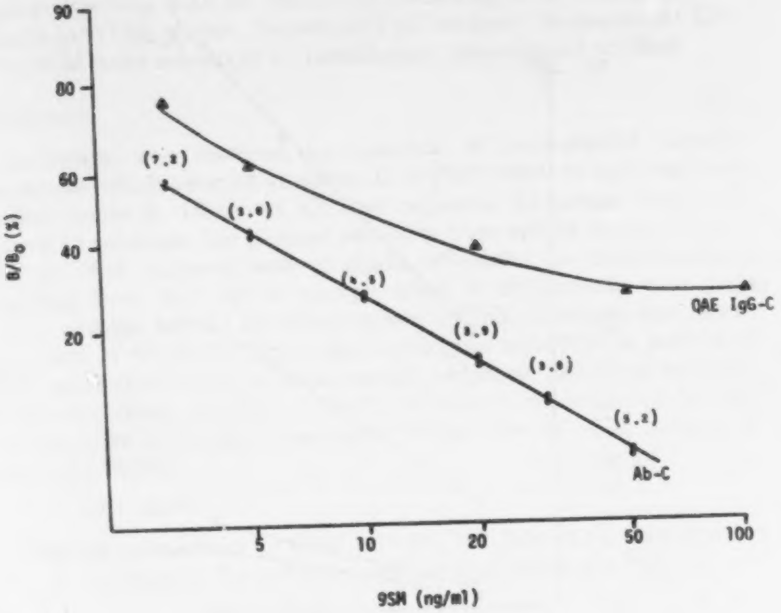


FIGURE 2. Solid-phase 9SM standard curves. THC-negative serum samples spiked with 9SM were added to tubes containing antibody-matrix conjugate and ^{3}H-Δ^9-THC. Matrix was counted after incubation with (B) and without (B$_0$) hapten. Log-logit transformations of mean values from six replicate assays were used to construct standard curves. The coefficient of variation for each point of the linear Ab-C standard curve is in parenthesis. The flattening of the QAE IgG-C curve at high 9SM concentrations is indicative of high capacity nonspecific binding.

In contrast, QAE IgG-C standard curves were nonlinear. This occurred because less antibody IgG was available for covalent linkage per unit volume of matrix in competition with other proteins in crude antibody fractions. Thus, larger aliquots of QAE IgG-C (100 µl) than Ab-C (10 µl) were required to bind sufficient ^{3}H-Δ^9-THC (figure 3). Resultant nonspecific binding caused extreme distortion of the QAE IgG-C standard curve resulting in loss of specificity and sensitivity.

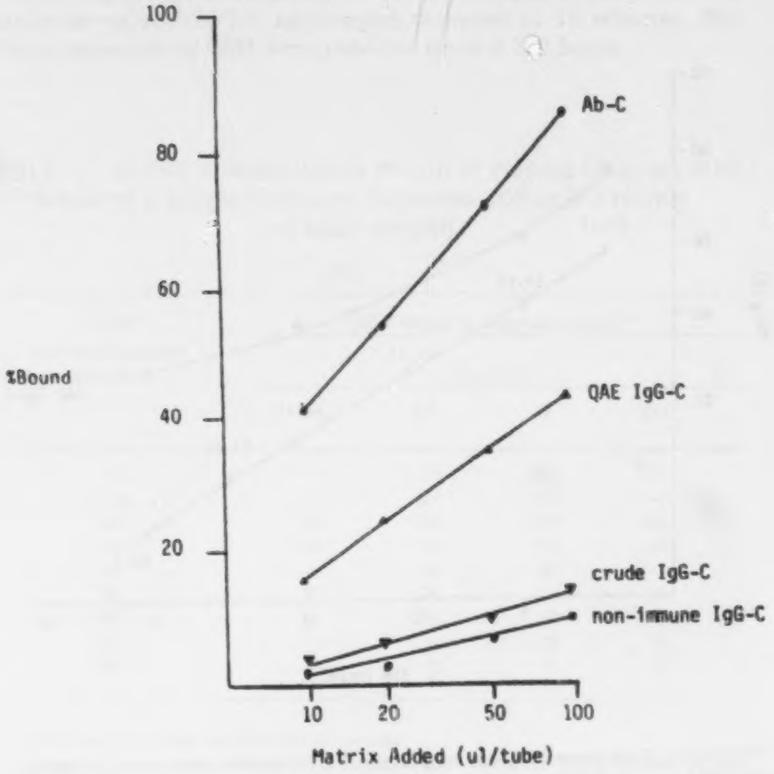


FIGURE 3. ^{3}H-Δ^9-THC binding to polyacrylate antibody conjugates. Binding of ^{3}H-Δ^9-THC to insolubilized antibody fractions of various purity. Small aliquots of immunosorbant-purified material (20 µl/30% v/v suspension) binds ^{3}H-Δ^9-THC well. The excessive amounts of QAE Sephadex antibody conjugate (100 µl/30% v/v suspension) needed for equivalent binding of ^{3}H-Δ^9-THC results in 30% nonspecific binding.

Precision

Intra-assay precision was measured by analysis of aliquots of single samples in the same assay. A mean coefficient of variation of 14% was obtained with four volunteers' sera assayed in duplicate. Inter-assay precision was evaluated by analysis of six samples in two separate assays. The coefficient of variation for these results was 17%.

Solid-Phase Dual-Metabolite Assay

Simultaneous quantitation of both Δ^9-THC and 9SM in a single assay tube was done by partitioning antibody into solid (9SM) and liquid (Δ^9-THC) phases. Soluble Δ^9-THC antibody measured Δ^9-THC. The 9SM were measured by homologous insolubilized antibody.

Accuracy

Accuracy was assessed by analysis of cannabinoid negative serum to which varying amounts of Δ^9-THC, 9SM, or both had been added (table 3). Observed (O) and expected (E) values were compared to calculate the percent recovery from spiked serum (O/E × 100%). 9SM recovery was excellent (92-120%) for concentrations ranging from 0-50 ng/ml except when Δ^9-THC levels were high (>10 ng/ml). In this situation excess Δ^9-THC displaces significant amounts of ^{3}H-Δ^9-THC from its homologous antibody. A portion of this label then binds to 9SM-specific antibody, resulting in slight underestimation of 9SM (70-84% recovery). Importantly, Δ^9-THC values were accurately measured irrespective of 9SM content of serum (92-110%).

TABLE 3. Recovery of 9SM and Δ^9-THC Added to Cannabinoid Negative Serum Measured by Simultaneous RIA

Cannabinoids added (ng/ml)		Cannabinoids measured (ng/ml)		Recovery (%)	
Δ^9-THC	9SM	Δ^9-THC	9SM	Δ^9-THC	9SM
0	10	0	10	—	100
0	25	0	30	—	120
0	50	0	55	—	110
5	0	5	0	—	—
5	10	5	10	100	100
5	25	5	23	100	100
5	50	5	45	100	92
10	0	9	0	90	—
10	25	11	20	110	80
10	50	10	42	100	84
20	0	24	0	120	—
20	50	22	35	92	70

Aliquots of serum were spiked with varying amounts of Δ^9-THC, 9SM, or both. These were assayed in a single test tube by solid-phase methods.

Subject Samples

9SM and Δ^9-THC were measured by this method in the sera of volunteer smokers. Liquid-phase assay was used to corroborate accuracy. Results from six subjects illustrate several important features (table 4). 9SM levels were precisely quantitated and were 4-6 times higher than corresponding Δ^9-THC values. Additionally, in certain subjects (3 and 5) 9SM were useful in confirming a marginal Δ^9-THC. Furthermore, the negative status of one control subject (4) with a reported Δ^9-THC of 2.3 ng/ml was affirmed by the absence of measurable 9SM.

TABLE 4. Sera Assayed for 9SM and Δ^9-THC by Simultaneous RIA

Subject	Standard RIA Results (ng/ml)		Single Tube Assay Results (ng/ml)	
	Δ^9-THC	9SM	Δ^9-THC	9SM
1	25	26	24	32
2	6	29	5	22
3	3	12.5	4.5	16
4	2.3	—	3.5	0
5	7.0	36.3	10	45
6	0	0	0	0

Marijuana metabolites were measured in subjects' sera within three hours of acute exposure. Standard liquid assay was used to assess the accuracy of simultaneous metabolite measurements.

DISCUSSION

The early liquid-phase RIA is a flexible technique which has been effectively employed for Δ^9-THC and metabolite measurements in minute volumes of body fluids using samples ranging from clear serum to hemolyzed blood. However, expertise in pipetting small volumes containing high concentrations of troublesome macromolecules (5) is required to quantitate low nanogram/ml concentrations. In addition, physical (charcoal, ammonium sulfate) or immunologic (second antibody) separation steps require critical timing and may introduce dilution, "stripping" and nonspecific binding errors. These limitations of liquid-phase RIA's have stimulated development of solid-phase techniques in many laboratories here and abroad. An antibody-coupled solid matrix simplifies and can even eliminate the steps necessary to separate free from bound hapten. Finally, since the solid phase is counted, water and macromolecule quenching of scintillant is avoided.

Reactive solid polymers can selectively remove and concentrate desired hapten from a dilute body fluid containing a great mixture of ingredients. Thus, such a matrix permits the collection and subsequent measurement of cannabinoids from breath samples.

It should be noted in this context that studies in which tritiated Δ^9-THC was administered intravenously (Mario Perez-Reyes, personal communication), no radioactivity was detected in saliva. If the present findings reflect metabolic processes at the lung surface, cannabinoid measurement in breath has potential significance.

The newer methods employing solids available in geometric forms ranging from regular tubes to amorphous beads lead to simultaneous serum Δ^9-THC and 9SM measurements in a single container. This has potential merit in gauging marijuana impairment, particularly when Δ^9-THC concentration is marginal. Long-term stability evaluations of purified antibody and the other solid reagents are now required. Polyphase supports will eventually encompass a large variety of molecules currently assayed by qualified technicians in hospital and reference laboratories. The new generation of methods will simplify their tasks and perhaps allow quantification in the low nanogram/ml range by less experienced individuals.

REFERENCES

1. S.J. Gross. Specificities of steroid antibodies, chapter 3, in *Immunologic Methods in Steroid Determination*, Appleton-Century-Crofts, 41-54 (1970).
2. J.D. Grant, S.J. Gross, P. Lomax, and R. Wong. Antibody detection of marijuana. *Nature New Biology, 236*, 216-217 (1972).
3. J.D. Grant, J.R. Soares, and S.J. Gross. Separation of high affinity hapten specific and crossreacting IgG population. *Immunochemistry, 12*, 481-484 (1975).
4. D. Rodbard, W. Bridson, and P.L. Rayford. Rapid calculation of radioimmunoassay results. *J. Lab. Clin. Med., 74*, 770-781 (1969).
5. R.S. Yalow and S.A. Berson. Problems of validation of radioimmunoassays, chapter XV, in *Principles of Competitive Protein Binding Assays*, J.P. Lippincott, 374-400 (1970).

AUTHORS

J. R. Soares, J. D. Grant, and S. J. Gross
Receptor Research Institute
1707 Gardena Avenue
Glendale, CA 91204

Analysis of Cannabinoids
Research Monograph 42, R. Hawks, Editor
National Institute on Drug Abuse, 1982

Combined High-Performance Liquid Chromatography and Radioimmunoassay Method for the Analysis of Δ^9-Tetrahydrocannabinol and its Metabolites in Plasma and Urine

Anthony C. Moffat, Peter L. Williams, and Laurence J. King

A high-performance liquid chromatography-radioimmunoassay method for the measurement of cannabinoids in plasma and urine is described. The experimental procedure consists of chromatographing a plasma extract or hydrolysed urine sample by high-performance liquid chromatography and quantifying the eluting cross-reacting cannabinoids with radioimmunoassay. The concentrations of Δ^9-tetrahydrocannabinol (THC), cannabinol, mono-hydroxylated metabolites, di-hydroxylated metabolites, Δ^9-THC-11-oic acid, and Δ^9-THC-11-oic acid ester glucuronide may be measured by this technique.

INTRODUCTION

Radioimmunoassays (RIA) for the analysis of cannabinoids in human body fluids have been described (1-5) and have a number of advantages compared with other analytical techniques. Biological fluids may be analysed without pretreatment, the sensitivity of the method is high, large numbers of samples may be analysed at the same time and the selectivity of the antiserum for cannabinoids gives the assay a high degree of specificity. However, each antiserum cross-reacts with different cannabinoids to different extents and these cross-reactions may be further changed by altering the assay conditions, radioligand, etc. (6). An antiserum for use in a RIA can be produced such that it may be specific for Δ^9-tetrahydrocannabinol (THC). Thus, THC levels in plasma may be measured directly with the use of this antiserum, even though other cannabinoids or THC metabolites may be present. However, if the 11-nor-Δ^9-THC-9-carboxylic acid (THC-11-oic acid) is to be measured in urine, then a separate antiserum, and hopefully one specific to the acid metabolite and no other, must be prepared for use in another RIA (7).

An alternative approach is to produce an antiserum which cross-reacts with a number of cannabinoids including THC and some of its metabolites. This antiserum may then be used to assay plasma or urine, but the levels found can only be expressed as cross-reacting cannabinoids, since the analytical results are the sum of all the cross-reacting cannabinoids present in the sample (8). Although only one RIA is now needed for any biological fluid to be assayed, the ease of analysis has been exchanged for lack of specificity.

Other methods for the assay of cannabinoids in biological fluids have been developed, but nearly all require lengthy analysis times, due partly to the extensive purification procedures required prior to the instrumental analysis. The use of chromatographic methods has aided analyses because of the separating power of the techniques, but detection problems have always been evident. Various devices have been used to increase the selectivity of ultra-violet (UV) detectors in high-performance liquid chromatographic (HPLC) analyses, e.g., by the use of a Sephadex column clean-up prior to HPLC (9) or by the use of simultaneous dual wavelength UV detection (10). However, the great separating power of HPLC has constantly been hindered by the lack of a specific detector. The use of HPLC as a clean-up procedure before the use of another analytical technique has obvious advantages, and methods involving mass spectrometry (MS) (11), gas chromatography (GC) using electron capture detection (12), and liquid scintillation counting for radiolabelled metabolites (13) have been used.

The combination of RIA with HPLC has obvious advantages. The drawback of the lack of a sensitive and specific detector for cannabinoids in HPLC can be overcome by the use of a radioimmunoassay, and the lack of complete specificity of an antiserum can be overcome by the prior use of HPLC. Thus, cross-reacting components can be separated by the HPLC system, and by collecting fractions at the end of the column the individual cannabinoids can be quantified. This procedure has been used for the quantification of THC and its metabolites in human plasma (14) and also for the analysis of THC metabolites in human urine (15). Plasma levels of THC obtained by the HPLC-RIA method have also been compared with those obtained by a GC-MS procedure. The HPLC-RIA methods are presented below, together with a discussion on the advantages of the combined technique for use in forensic toxicology.

COLLECTION OF SAMPLES

Volunteers smoked tobacco cigarettes impregnated with either 10 or 8 mg of THC over a 10-minute period. Blood samples (10 ml) were taken at timed intervals after the subject had finished smoking. Anticoagulant (potassium EDTA, 10 mg) was added to the blood, which was centrifuged to separate the plasma. Urine samples from the same subjects were collected in silanised glass containers during the 24-hour period after smoking. Control urine samples were obtained from volunteers with no experience of cannabis use. All samples were stored at -20°C until analysis.

RADIOIMMUNOASSAY

Antiserum (133Y/22/5) for the assay was obtained from Dr. J. D. Teale, Department of Biochemistry, University of Surrey, Great Britain. Δ^9-THC and other cannabinoid compounds were provided by the National Institute on Drug Abuse, Rockville, Maryland, U.S.A. Δ^9-(G-^{3}H)-THC was purchased from the Radiochemical Centre, Amersham, Great Britain, polyvinylpyrrolidone-40 and charcoal (Norit A) from Sigma, St. Louis, Missouri, U.S.A., Dextran T70 from Pharmacia, Uppsala, Sweden, and methanol (AnalaR) and all other chemicals and solvents were obtained from BDH, Poole, Great Britain. An Intertechnique SL30 was used for liquid scintillation counting.

Antiserum (133Y/22/5) was stored as aliquots in buffer (0.1M phosphate buffer pH 7.5, containing 0.2 percent polyvinylpyrrolidone-40) at -20°C and diluted to 1:300 before use. Solutions of Δ^9-THC used to calibrate the assay were made up in aqueous methanol (50 percent v/v) at concentrations ranging from 500 pg/ml to 50 ng/ml and stored at -20°C. Δ^9-(^{3}H)-THC (12 Ci/mmole) was also stored at -20°C at a concentration of 0.25 μCi/ml in aqueous methanol (50 percent v/v, pH 8.6) ready for use.

The plasma sample to be assayed was mixed with three volumes of methanol, vortexed and allowed to stand for 30 min. It was then centrifuged and the supernatant added directly to the assay tubes. Normal human plasma similarly treated was used in the total, nonspecific binding and zero binding tubes and for assay dilutions. The plasma extract (100 μl) was mixed with the radiolabel (50 μl), 50 percent v/v methanol (100 μl), and antiserum (100 μl). The final assay volume was made up to 600 μl with buffer and methanol to give a final methanol concentration of 25 percent v/v. After mixing, the solutions were left to stand at room temperature for 1 hour. Dextran-coated charcoal (200 μl, pH 7.4) was added, the tubes were centrifuged, and after 2 minutes contact time the supernatant (500 μl) from each tube was counted. Urine samples were treated in a similar manner. All analyses were performed in duplicate.

HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY-RADIOIMMUNOASSAY

A constant-flow pump (M-6000A Waters Associates, Milford, Massachusetts, U.S.A.) was used to deliver a methanol-water eluent to a stainless-steel HPLC column (10 cm × 4.6 mm i.d.) slurry-packed with Spherisorb-5-ODS (Phase Separations, Flintshire, Great Britain). Samples were introduced into the HPLC column with a six-port injection valve (Spectroscopy Accessory, Sidcup, Great Britain) fitted with a 10 ml injection loop. Column eluate was either monitored with an ultraviolet (UV) detector at 280 nm (Cecil CE212) or collected with a Struers Samplomat fraction collector (Camlab, Cambridge, Great Britain). A freezedrier (Model SB4, Chemlaboratory Instruments, Ilford, Great Britain) was used to remove solvents.

Plasma (0.2-1 ml) was mixed with three volumes of methanol, vortexed, left to stand for 30 min and centrifuged. The supernatant was removed, the residue mixed with methanol and the sample again centrifuged. Water was added to the combined supernatants to give a methanol concentration of 50 percent (v/v). Urine (0.1-3 ml) was mixed with methanol and a methanol-water (50:50) buffer (0.1M acetic acid adjusted to pH 5.5 with sodium hydroxide) to give a final volume of 6 ml and a methanol concentration of 50 percent v/v. Metabolite conjugates in urine were hydrolysed by adding an equal volume of methanolic sodium hydroxide (1M) to the urine, evacuating the vessel to remove oxygen and leaving at room temperature for 30 minutes. Sufficient acetic acid was then added to give a pH of 5.5 and the volume was made up to 6 ml with methanol-water (50:50) buffer (pH 5.5).

Plasma extract and unhydrolysed or hydrolysed urine samples treated as above were then injected onto the HPLC column with the loop injector. A stepped solvent elution programme was used:

10 ml of methanol-water (50:50), 10 ml of methanol-water (62.5:37.5), 20 ml of methanol-water (72.5:27.5) at a flow rate of 1 ml/min. Eluate fractions were taken every 30 seconds and were lyophilised. The freezedrier was flushed with argon before and after drying to prevent atmospheric oxidation of sensitive metabolites. A solution of Δ^9-(^{3}H)-THC (0.025 μCi in 500 μl of a mixture of methanol-diluent buffer, 30:70) was added to each of the dried tubes with a solution of antiserum in diluent buffer (100 μl of 1:300 antiserum solution). The nonspecific binding tubes received diluent buffer in place of antiserum solution. The subsequent RIA procedure was the same as that described above.

Cannabinoid retention volumes were determined by monitoring the eluate with either RIA for cross-reacting compounds, or UV absorption (280 nm) for non-cross-reacting compounds. UV detection required μg quantities compared with the ng quantities needed for RIA detection.

GAS CHROMATOGRAPHY-MASS SPECTROMETRY

The internal standard (5′,5′,5′-^{2}H$_3$-Δ^9-THC, 10 ng) in methanol was added to the plasma sample (1 ml) and the mixture extracted with methanol as above. The plasma extract was chromatographed using the HPLC column above and an eluent of methanol-water (67.5:32.5). The THC fraction (retention volume 20 ml) was collected, lyophilised and the residue dissolved in hexane. Injections were made into a Pye 104 GC using a 3 percent OV-17 column (0.3 m × 2 mm i.d., Gas Chrom Q, 80-100 mesh) at 190°C when THC had a retention time of 4 min with a helium flow rate of 20 ml/min. The VG Micromass 16F mass spectrometer was used in the multiple ion detection mode tuned to ions of m/z 299 and 314 for THC and 302 and 317 for the tri-deuterated THC. Plasma concentrations of THC were calculated from the ratio of ion intensities for THC and internal standard using a previously prepared calibration graph.

RESULTS AND DISCUSSION

The antiserum used in this study did not cross-react with 44 non-cannabinoid drugs at concentrations of 400 μg/ml in plasma and is therefore very selective for cannabinoids. It does, however, cross-react with a number of cannabinoids (table 1). Minor modifications to the molecule at the 8, 9, or 11 positions do not alter the cross-reactivity significantly, so the antiserum can be used to measure THC, or any of the metabolites formed by changes at the 8, 9, or 11 positions, in either plasma or urine. The level of cross-reaction normally obtained for blank plasma is in the region of 4 ng/ml, with only exceptional cases being as high as 9 ng/ml. The supernatant serum from a post mortem specimen of blood which is grossly haemolysed gives similar values, so that a suitable cut-off level for a positive result is about 5 ng/ml. Urine samples have a much lower cut-off level (1 ng/ml) and only in exceptional circumstances, e.g., if a large amount of an antibacterial inorganic preservative is added, may the level be above this.

TABLE 1. Cross-Reactivity of Δ^9-THC Metabolites and Analogues in the Radioimmunoassay

Cannabinoid	Amount required for 50% depression of binding (ng)
Δ^9-THC	0.6
Δ^8-THC	0.6
CBN	0.6
11-Hydroxy-Δ^9-THC	0.6
Δ^9-TCH-11-oic acid	0.6
11-Hydroxy-CBN	0.6
CBN-11-oic acid	0.6
Hexahydro-CBN	0.6
$\Delta^{9,11}$-THC (Exo-cyclic compound)	0.6
8α-Hydroxy-Δ^9-THC	0.6
8β-Hydroxy-Δ^9-THC	3.0
8α,11-Dihydroxy-Δ^9-THC	2.0
8β,11-Dihydroxy-Δ^9-THC	9.0
1′-Oxo-CBN	3.0
1′-Hydroxy-CBN	10
5′-Hydroxy-Δ^9-THC	30
CBD	>50
Cannabicyclol	>50
Cannabichromene	>50
Cannabigerol	>50

The plasma levels of cannabinoids, determined by direct RIA, for volunteers who smoked 10 mg THC were between 70 and 6 ng/ml over the 2-hour period examined. Table 2 gives the results for one smoker who was a cannabis user and had THC metabolites in his plasma before the experiment started.

TABLE 2. Plasma Concentrations of Δ^9-THC in a Subject Who Had Smoked 10 mg Δ^9-THC Measured by RIA, HPLC-RIA and GC-MS

Time after smoking (min)	Plasma concentration of Δ^9-THC (ng/ml)		
	Direct RIA*	GC-MS	HPLC-RIA
0	23**	0	0
2	67	55	47
12	48	18	15
24	47	9	7
34	48	8	5
64	47	5	3
126	48	2	1

* These values include contributions from THC metabolites.

** The subject was a cannabis user and this level represents the level of cannabinoids present in his plasma before the experiment started.

The results obtained by the GC-MS method indicate that no THC was present at the start of the experiment and a high level (55 ng/ml) was obtained 2 minutes after stopping smoking. The level gradually fell to 2 ng/ml over the 2-hour period. The direct RIA results, however, do not show the same fall, since at 2 hours the plasma still had a level of 48 ng/ml cross-reacting cannabinoids. Much of the THC had left the plasma by this time, but there was still an appreciable level of its metabolites cross-reacting in the RIA.

The HPLC-RIA method can separate and individually quantify these cross-reacting metabolites, and figure 1 shows a HPLC-RIA chromatogram of a plasma sample taken 13 minutes after the volunteer had finished smoking a cigarette containing THC. Although there was still some THC present, there were also cross-reacting compounds eluting at the retention volumes of the mono- and dihydroxylated metabolites, with the greatest contribution coming from the compounds with the elution volume 10.0 ml, which correspond to the THC-11-oic acid and its ester glucuronide.

The retention volumes of THC, its metabolites, and other cannabinoids are given in table 3. The quantity of any of the compounds may be obtained by integrating the individual points on the peak

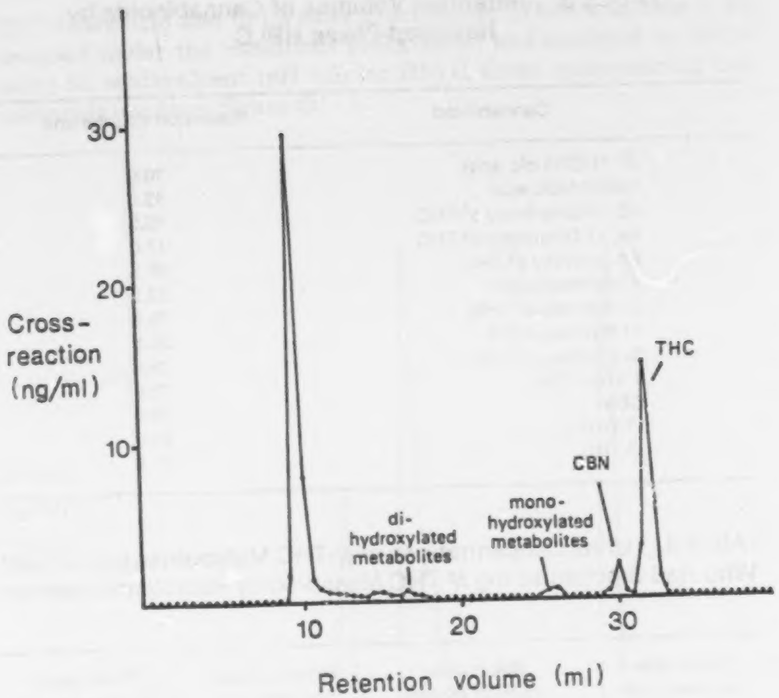


FIGURE 1. HPLC-RIA chromatogram of a plasma sample from a subject taken 13 minutes after smoking 10mg Δ^9-THC. The retention volumes of THC, CBN, the mono-hydroxylated metabolites and di-hydroxylated metabolites are marked.

to give the total contribution for that compound. Thus, when the HPLC-RIA method is used to quantify the THC in the plasma samples, very good agreement with the GC-MS results is obtained (table 2), since both methods are now measuring the same compound (THC) without any other cannabinoids interfering in the assay.

The detection of cannabis smoking by an individual who habitually uses cannabis is very much easier when urine is analysed by RIA because the levels of cross-reacting cannabinoids are much higher than in plasma. For example, a single smoked dose of 10 mg THC still gave levels of 49 ng/ml cross-reacting cannabinoids in the urine 24 hours after smoking (table 4), although it must be noted

TABLE 3. Retention Volumes of Cannabinoids by Reversed-Phase HPLC

Cannabinoid	Retention volume (ml)
Δ^9-THC-11-oic acid	10.0
CBN-11-oic acid	10.0
8β, 11-Dihydroxy-Δ^9-THC	16.5
8α, 11-Dihydroxy-Δ^9-THC	17.0
8β,-Hydroxy-Δ^9-THC	20.5
1′-Hydroxy-CBN	22.5
11-Hydroxy-Δ^9-THC	25.5
11-Hydroxy-CBN	25.5
8α-Hydroxy-Δ^9-THC	25.5
1′-Oxo-CBN	29.0
CBN	30.0
Δ^8-THC	31.0
Δ^9-THC	31.5

TABLE 4. Urine Concentrations of Δ^9-THC Metabolites in a Subject Who Had Smoked 10 mg Δ^9-THC Measured by Radioimmunoassay

Time after smoking (hr)	RIA cross-reaction (ng/ml)	Urine volume (ml)	Total cross-reacting material (μg/hr)
0	35*	132	—
1	117	111	13.0
2	142	34	4.8
3	125	56	7.0
4	145	44	6.4
6	135	38	2.6
8	78	113	4.4
24	49	600	1.8

*The subject was a cannabis user; this level represents the level of cannabinoids present in his urine before the experiment started.

that this subject, a cannabis user, started the experiment with a level of 35 ng/ml.

When an unhydrolysed urine sample is chromatographed under neutral conditions and the eluate analysed by HPLC, only one peak is seen, which has a retention volume of 10 ml. This corresponds to

THC-11-oic acid and to its ester glucuronide. When a sample is hydrolysed under the conditions given above and analysed by HPLC using an acidic eluent (pH 5.5) for HPLC, three cross-reacting cannabinoids are seen (figure 2).

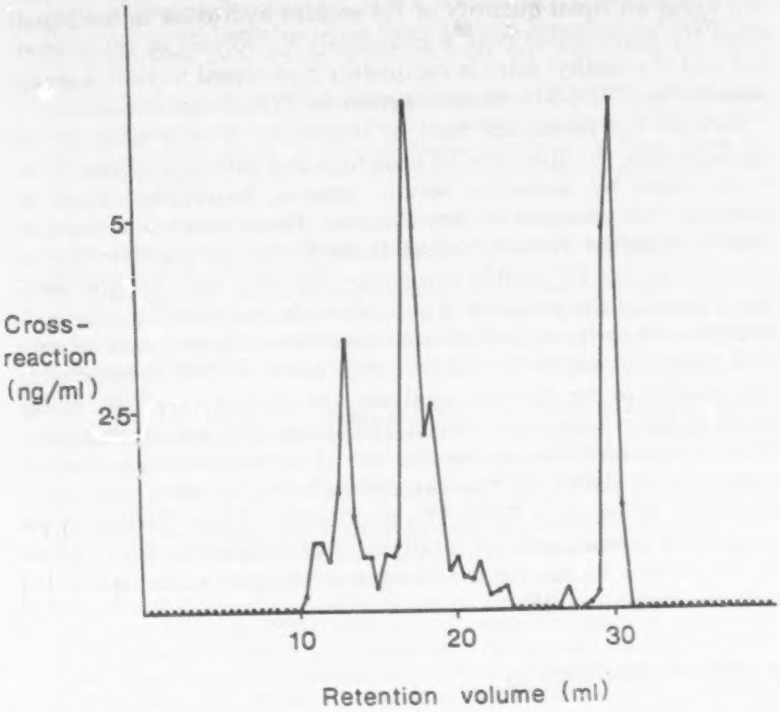


FIGURE 2. HPLC-RIA chromatogram of a hydrolysed urine sample from an individual suspected of having smoked cannabis.

The first peak (retention volume 13.5 ml) is the glucuronide ester of THC-11-oic acid, the second peak (retention volume 16.5 ml) is THC-11-oic acid, and the third peak (retention volume 30 ml) is the methyl ester of THC-11-oic acid. The identities of all three peaks have been established by GC-MS. The predominant THC metabolite in urine, as detected by the antiserum used in this work, is therefore the glucuronide ester of THC-11-oic acid. Under the conditions of hydrolysis used above, this glucuronide is transesterified to the methyl ester of THC-11-oic acid, which is then hydrolysed to the free acid. The three peaks may be used as confirmation of the presence of cannabinoids in the urine sample in the same way as derivatives are used for proof of identity in GC analyses. In some cases a quicker analysis for urine may be required, in which case the hydrolysis conditions may be made more vigorous (100°C for 15 min using an equal quantity of 1M sodium hydroxide in methanol) when the glucuronide peak is completely hydrolysed or transesterified and the methyl ester is completely hydrolysed to yield a single peak in the HPLC-RIA chromatogram for THC-11-oic acid (16).

Because the antiserum used in this study is very selective for cannabinoids, the RIA may be used to detect cannabis misuse in forensic cases by screening serum, plasma, haemolysed blood or urine for the presence of cannabinoids. Those which are negative may be reported as such, whilst those which are positive may be analysed by the HPLC-RIA procedure. Not only will this give additional proof of the presence of cannabinoids, but it will enable each cannabinoid to be identified and quantified. Three years' experience using this method has shown that levels of THC in blood samples submitted for forensic analyses are usually very low, whilst levels of the glucuronide ester of THC-11-oic acid are much higher. This is an added reason for the use of a relatively nonselective antiserum to detect all the cannabinoids whilst using the HPLC procedure to separate them. The application of this method to the analysis of cannabinoids in fatally injured drivers has been reported (17), as has its use for the analysis of samples submitted for forensic examination (18).

ACKNOWLEDGMENTS

We thank Professor J. W. Thompson (University of Newcastle upon Tyne) for supplying plasma and urine samples from THC smokers; Dr. S. Agurell and Ms. A. Ohlsson (Astra Lakemedel, Sodetalje, Sweden) for the samples of deuterated THC; Dr. J. D. Teale (University of Surrey) for the antisera; and Dr. R. E. Willette (National Institute on Drug Abuse) for samples of cannabinoids and their metabolites.

REFERENCES

1. J.D. Teale, E.J. Forman, L.J. King, and V. Marks. Production of antibodies to tetrahydrocannabinol as the basis for its radioimmunoassay. *Nature* (London), *249*, 154-155 (1974).

2. J.D. Teale, E.J. Forman, L.J. King, E.M. Piall, and V. Marks. The development of a radioimmunoassay for cannabinoids in blood and urine. *J. Pharm. Pharmacol., 27,* 465-472 (1975).
3. S.J. Gross, J.R. Soares, S-L.R. Wong, and R.E. Schuster. Marihuana metabolites measured by a radioimmune technique. *Nature* (London), *252,* 581-582 (1974).
4. C.E. Cook, M.L. Hawes, E.E. Amerson, C.G. Pitt, and D. Williams. Radioimmunoassay of Δ^9-tetrahydrocannabinol. In R.E. Willette (ed.), *Cannabinoid Assays in Humans,* National Institute on Drug Abuse Research Monograph 7, DHEW Pub. No. (ADM)76-339, Supt. of Docs., U.S. Govt. Print. Off., Washington, D.C., 15-27 (1976).
5. A.R. Chase, P.R. Kelley, A. Taunton-Rigby, R.T. Jones, and T. Harwood. Quantitation of cannabinoids in biological fluids by radioimmunoassay. In R.E. Willette (ed.), *Cannabinoid Assays in Humans,* National Institute on Drug Abuse Research Monograph 7, DHEW Pub. No. (ADM)76-339, Supt. of Docs., U.S. Govt. Print. Off., Washington, 1-9 (1976).
6. C.E. Cook. Radioimmunoassay of cannabinoid compounds. In J.A. Vinson (ed.), *Cannabinoid Analysis in Physiological Fluids,* American Chemical Society, Washington, D.C., 137-154 (1979).
7. S.J. Gross and J.R. Soares. Separate radioimmune measurements of body fluid Δ^9-THC and 11-nor-9-carboxy-Δ^9-THC. In R.E. Willette (ed.), *Cannabinoid Assays in Humans,* National Institute on Drug Abuse Research Monograph 7, DHEW Pub. No. (ADM)76-339, Supt. of Docs., U.S. Govt. Print. Off., Washington, D.C., 10-14 (1976).
8. J.D. Teale, J.M. Clough, L.J. King, V. Marks, P.L. Williams, and A.C. Moffat. Antisera raised against tetrahydrocannabinol in the radioimmunoassay of cannabinoids. In J.A. Vinson (ed.), *Cannabinoid Analysis in Physiological Fluids,* American Chemical Society, Washington, D.C., 155-173 (1979).
9. J.L. Valentine, O.H.M. Gan, H.C. Nio, and E.D. Thompson. HPLC analyses of Δ^9-tetrahydrocannabinol and 11-nor-Δ^9-tetrahydrocannabinol-9-carboxylic acid in human plasma. In J.A. Vinson (ed.), *Cannabinoid Analysis in Physiological Fluids,* American Chemical Society, Washington, D.C., 175-205 (1979).
10. S.R. Abbot, J.R. Berg, K.O. Loeffler, S. Kanter, L.E. Hollister, J.H. Abrams, H.L. Baras, and R.T. Jones. HPLC analysis of Δ^9-tetrahydrocannabinol and metabolites in biological fluids. In J.A. Vinson (ed.), *Cannabinoid Analysis in Physiological Fluids,* American Chemical Society, Washington, D.C., 115-136 (1979).
11. J.L. Valentine, P.J. Bryant, P.L. Guttshall, O.H.M. Gan, E.D. Thompson, and H.C. Niu. HPLC-MS determination of Δ^9-tetrahydrocannabinol in human body fluids. In R.E. Willette (ed.), *Cannabinoid Assays in Humans,* National Institute on Drug Abuse Research Monograph 7, DHEW Pub. No. (ADM)76-339, Supt. of Docs., U.S. Govt. Print. Off., Washington, D.C., 96-106 (1976).
12. E.R. Garrett, A.J. Gouyette, and C.A. Hunt. GLC and HPLC analyses of cannabinoids in biological fluids and applications. In J.A. Vinson (ed.), *Cannabinoid Analysis in Physiological Fluids,* American Chemical Society, Washington, D.C., 13-37 (1979).
13. E.R. Garrett and C.A. Hunt. Separation and sensitive assay of THC in biological fluids by HPLC and GLC. In R.E. Willette (ed.), *Cannabinoid Assays in Humans,* National Institute on Drug Abuse Research Monograph 7, DHEW Pub. No. (ADM)76-339, Supt. of Docs., U.S. Govt. Print. Off., Washington, D.C., 33-41 (1976).
14. P.L. Williams, A.C. Moffat, and L.J. King. Combined high-performance liquid chromatography and radioimmunoassay method for the quantitation of Δ^9-tetrahydrocannabinol and some of its metabolites in human plasma. *J. Chromatogr., 155,* 273-283 (1978).

15. P.L. Williams, A.C. Moffat, and L.J. King. Combined high-performance liquid chromatography and radioimmunoassay method for the analysis of Δ^9-tetrahydrocannabinol metabolites in human urine. *J. Chromatogr., 186,* 595-603 (1979).
16. B. Law. Unpublished observations.
17. J.D. Teale, J.M. Clough, L.J. King, V. Marks, P.L. Williams, and A.C. Moffat. The incidence of cannabinoids in fatally injured drivers: an investigation by radioimmunoassay and high pressure liquid chromatography.*J. Forens. Sci. Soc., 17,* 177-183 (1977).
18. B. Law, P.L. Williams, and A.C. Moffat. The detection and quantification of cannabinoids in blood and urine by RIA, HPLC/RIA and GC/MS. *Vet. and Human Toxicol., 21,* 144-147 (1979).

AUTHORS

Anthony C. Moffat and Peter L. Williams
Home Office, Central Research Establishment, Aldermaston,
Reading, Berks RG7 4PN, Great Britain

Laurence J. King
Department of Biochemistry
University of Surrey, Guildford,
Surrey GU2 5XH, Great Britain

British Crown Copyright

Analysis of Cannabinoids
Research Monograph 42, R. Hawks, Editor
National Institute on Drug Abuse, 1982

An EMIT Assay for Cannabinoid Metabolites in Urine

M. J. DeLaurentis, K. McNeil, A. J. Mann, S. Clark, and Helena M. Greenwood

INTRODUCTION

An assay to detect cannabinoid metabolites in urine has been developed for application as a screening test for the use of marijuana. The technique employed was homogeneous enzyme immunoassay (EMIT), a non-isotopic immunoassay method first described for the measurement of morphine in urine (1). The immunoassay employs an enzyme as label. When the antigen to be assayed is conjugated to an enzyme, the enzyme activity may be modified by antibody binding. This forms the basis of the assay, where enzyme activity is directly proportional to the free antigen concentration. Four enzymes have been employed in the development of assays for drugs, both in urine and serum, for endogenous haptens and more recently for serum proteins; these applications of the technique have been reviewed elsewhere (2,3).

Further application of the technique, using MDH conjugated with a derivative of Δ^9-tetrahydrocannabinol (Δ^9-THC), has demonstrated that the procedure is equally useful in the determination of cannabinoids in urine. Pig heart mitochondrial malate dehydrogenase (MDH) E.C. 1.1.1.37 was chosen for development of a THC assay because of its availability and its ease of detection. This, together with antibodies raised in sheep, enabled development of a rapid, sensitive and specific assay where as little as 20 ng of THC together with its metabolites can be detected in a 1-minute spectrophotometric measurement.

ASSAY DEVELOPMENT

In order to develop an assay which would detect most commonly occurring cannabinoid metabolites present in urine, antibodies were raised against a molecule derivatized at a point common to many such metabolites. The principal metabolites believed to be present in urine are shown in figure 1. The desired acid (I) was

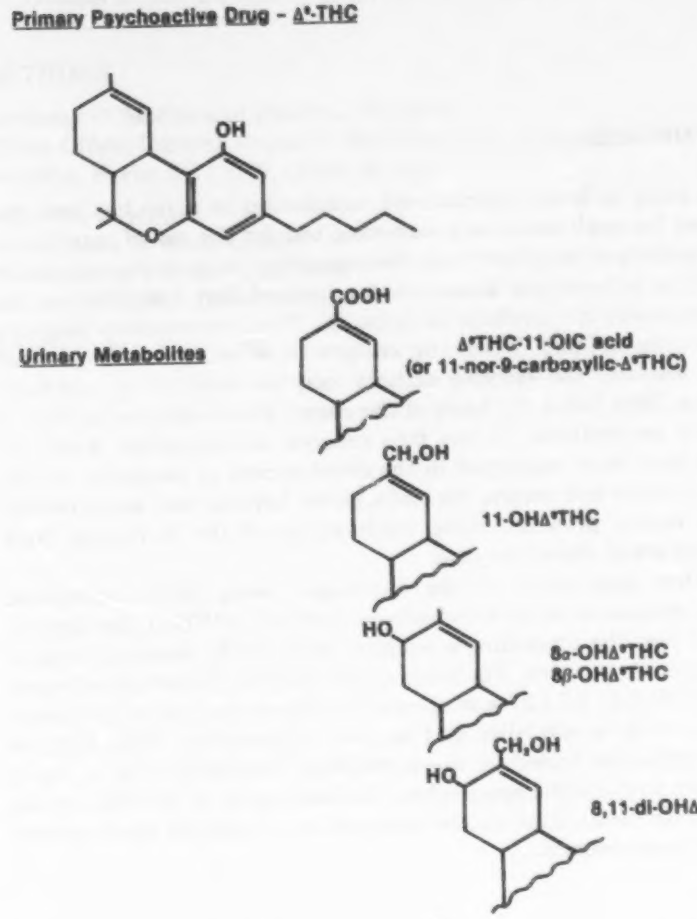
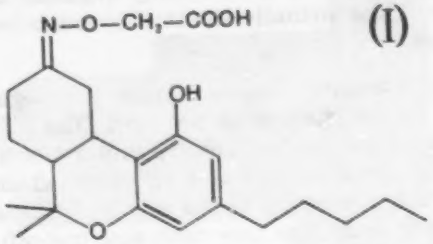

Primary Psychoactive Drug - Δ⁹-THC
OH
Urinary Metabolites
COOH
Δ⁹THC-11-OIC acid
(or 11-nor-9-carboxylic-Δ⁹THC)
CH₂OH
11-OHΔ⁹THC
HO
8α-OHΔ⁹THC
8β-OHΔ⁹THC
CH₂OH
HO
8,11-di-OHΔ⁹THC

FIGURE 1. Structures of Δ^9-THC and metabolites believed to be present in urine.

produced by the method previously described (4) and was conjugated to the enzyme, pig heart malate dehydrogenase. The mechanism of inhibition of hapten conjugates of MDH has been previously described in an immunoassay for morphine (5). Antibodies to the cannabinoid metabolites were obtained from sheep by immunization with a bovine gamma-globulin conjugate of the acid (I).



(I)

When the Δ^{9}-THC acid derivative was conjugated to MDH and mixed with an antibody excess, a 60% inhibition of enzyme activity resulted. This inhibition could be reversed by addition of increasing amounts of Δ^{9}-THC acid such that it was possible to develop an assay in which drug concentration is directly proportional to activity of the MDH conjugate. Using a spectrophotometer, the enzyme rate is followed at 340 nm by measuring the rate of conversion of the coenzyme NAD into NADH.

Assay Protocol

Using the Syva Model 1500 Pipetter/Diluter, 50 µl of urine sample is delivered to a sample cup with 250 µl of buffer which contains the enzyme substrate, malic acid. By sequentially adding 1) 50 µl of antibody reagent, which contains antibodies to a THC derivative and coenzyme nicotinamide adenine dinucleotide (NAD), together with 250 µl of buffer and 2) 50 µl of enzyme reagent plus 250 µl of assay buffer, the final reaction mixture is produced. The mixture is aspirated into the flowcell of a spectrophotometer, where the enzyme rate is measured at 30°C for 30 seconds following a 15-second delay. Enzyme activity is proportionally reduced when the labeled drug combines with antibody; the active enzyme converts NAD to NADH, resulting in an absorbance change that is measured spectrophotometrically at 340 nm.

Calibration

Three EMIT calibrators are employed in the assay; the Negative calibrator contains no drug (0.0 ng/ml) and the Low (20 ng/ml) and Medium (75 ng/ml) calibrators contain the drug derivative, 11-nor-Δ^9-THC-9-carboxylic acid. Since Δ^9-THC acid is difficult both to isolate and to prepare, Δ^8-THC was selected to act as calibrator for the assay. Δ^8-THC acid is more readily available than the Δ^9-THC acid, and it exhibits virtually identical immunochemical reactivity in this system.

For a positive/negative interpretation, the Negative (0.0 ng/ml) and Low (20 ng/ml) calibrators are used. The difference between the rate observed for the Negative calibrator and the rate observed for the Low calibrator is used as a reference which permits differentiation of urine samples containing no detectable drug from those containing drug. The Low calibrator (cut-off) contains a predetermined quantity of drug derivative which ensures that at least 95% of all samples that contain levels of cannabinoids equal to or in excess of the detection limit of the assay (50 ng/ml) yield a positive response, and at least 95% of samples containing no cannabinoids give a response less than that of the calibrator.

To test for the presence or absence of cannabinoids in urine, it was necessary to select this specific minimum reading (cut-off) above which samples can be identified as positive. For the EMIT Cannabinoid Assay, the response of the Low calibrator (nominal concentration 20 ng/ml) serves as the cut-off, since it was found to give a rate which discriminates effectively between samples containing 50 ng/ml and samples containing no drug. This was confirmed by assaying 50 samples known to be negative for THC, both before and after spiking each of them with 50 ng/ml Δ^8-THC acid. The results, when plotted as a histogram, show the separation between the two populations with no overlap between positive and negative samples (figure 2).

Alternatively, a standard curve (figure 3) for the assay can be prepared by plotting the assay reading (ΔA^* of the Negative, Low, and Medium calibrators against the concentration on semi-log graph paper. A semiquantitative result can then be estimated from the standard curve. Because of sample-to-sample variation and the fact that the assay detects certain cannabinoid metabolites and analogs with different sensitivity from that of the calibrator drug, semiquantitative results for positive samples can only be estimated in analyte equivalents.

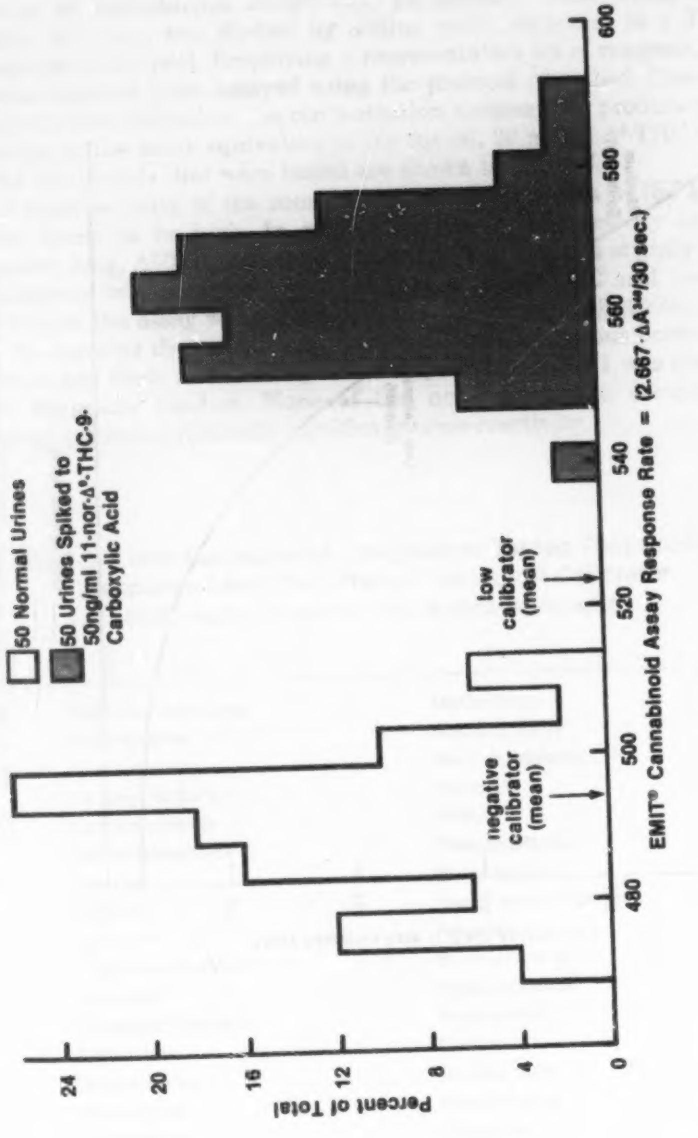


FIGURE 2. Histogram showing assay response of 50 THC negative samples and the same samples spiked with 50 ng/ml 11-nor-Δ^9-carboxylic acid.

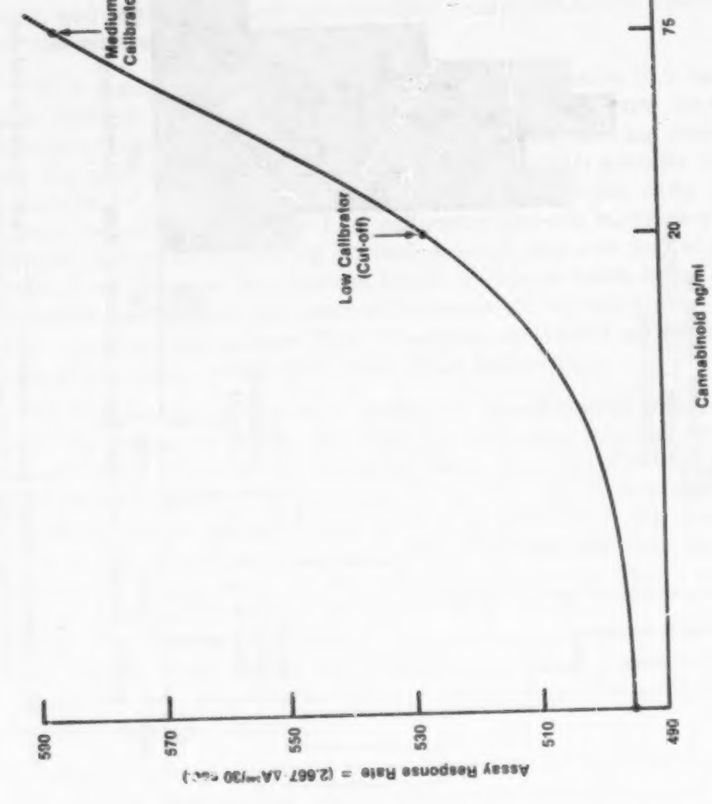


FIGURE 3. Response curve for the EMIT Cannabinoid Assay.

Specificity/Cross-Reactivity

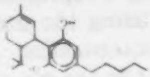
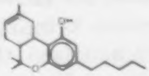
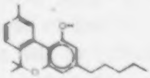
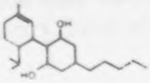
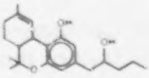
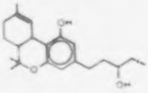
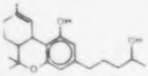
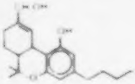
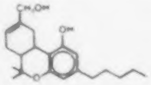
The cross-reactivity of the EMIT Cannabinoid Assay to a wide range of cannabinoid compounds, particularly those thought to occur in urine, was studied by adding each compound to a THC-negative urine pool. Employing a representative lot of reagents, the urine samples were assayed using the protocol described. Cross-reactivity was defined as the concentration necessary to produce a response in the assay equivalent to the cut-off, 20 ng/ml Δ^8-THC acid. The compounds that were tested are shown in figure 4.

Cross-reactivity of the common urinary metabolites in the assay was found to be high. In comparison the cross-reactivity to the parent drug, Δ^9-THC, which is not found in urine, was slightly less. Cross-reactivity between the Δ^9-THC acid and Δ^8-THC acid used to calibrate the assay was determined to be approximately 100%.

To examine the cross-reactivity of the assay to various hormones, drugs and their metabolites, each compound in table 1 was studied by the same method. None of the non-cannabinoid compounds tested exhibited clinically significant cross-reactivity.

TABLE 1. Non-Cannabinoid Compounds Tested That Produce a Response Less Than That of the Cutoff Calibrator (< 20 ng/ml 11-nor-Δ^8-THC-9-carboxylic acid)

Acetylsalicylic acid
Amitriptyline
Amphetamine
Benzoyl ecgonine
Carbamazepine
Chlordiazepoxide
Codeine
Cortisol
Demerol
Dextromethorphan
Diazepam
Diphenylhydantoin
Ephedrine
Glutethimide
Imipramine
Medazapan
Methadone
Methaqualone
Methylphenidate
Naloxone
PEMA
Phencyclidine
Phenobarbital
Phenylpropanolamine
Primidone
Promethazine
Propoxyphene
Propranolol
Quinidine
Secobarbital
Theophylline
Thorazine

Compound	Structure	Concentration Yielding Response Equivalent to 20ng/ml Δ^9-THC-9-Acid
Δ^9-THC		66ng/ml
Δ^8-THC		71ng/ml
Cannabinol (CBN)		191ng/ml
Cannabidiol (CBD)		1400ng/ml
2′-OH-Δ^9-THC		302ng/ml
3′-OH-Δ^9-THC		271ng/ml
4′-OH-Δ^9-THC		112ng/ml
11-OH-Δ^9-THC		19ng/ml
11-OH-Δ^8-THC		24ng/ml

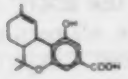
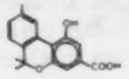
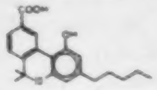
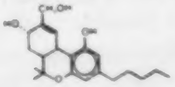
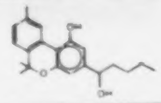
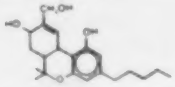
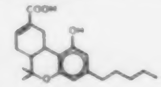
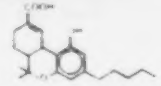
Compound	Structure	Concentration Yielding Response Equivalent to 20ng/ml Δ⁹-THC-9-Acid
1′2′3′4′5′ Penta-nor Δ⁹-THC-3-COOH		>10μg/ml
1′2′3′4′5′ Penta-nor CBN-3-COOH		>10μg/ml
11-nor-CBN-9-COOH		39ng/ml
8β-11-diOH-Δ⁹-THC		38ng/ml
1′-OH-CNB		>10μg/ml
8β-diOH-Δ⁹-THC		32ng/ml
11-nor-Δ⁸-THC-9-COOH		22.6ng/ml
11-nor-Δ⁹-THC-9-Acid		20ng/ml

FIGURE 4. Cross-reactivity of a representative reagent lot to various cannabinoid compounds.

CLINICAL STUDIES

Smoking Study

In a controlled study using the EMIT Cannabinoid Assay, three male and three female subjects, all regular users of marijuana, abstained from smoking for at least 6 days (10 days maximum). Each subject then smoked two marijuana cigarettes, each containing 9 mg THC, within a 2-hour interval. Urinary cannabinoid levels were monitored over the following 10-day period (figures 5 and 6) during which the subjects abstained from further marijuana use.

As can be seen from figures 5 and 6, the data from the controlled study indicate that urinary cannabinoid levels of regular users of marijuana, male and female, may be in excess of 20 ng/ml for 4 to 10 days and longer after smoking. Although the cannabinoid levels detected with the EMIT Cannabinoid Assay were highest at the beginning of the study, it is not possible to distinguish between recent use (within 24 hours) and infrequent use (up to 10 days).

Specificity Study

To evaluate the assay for potential cross-reaction due to non-THC drug metabolites, clinical investigators were asked to assay as many samples as possible that were known to contain drugs or drug metabolites other than THC (figure 7). The presence or absence of cannabinoids in these samples was not confirmed by a non-immunochemical technique.

As can be seen from the figure, there appears to be no correlation between samples positive for cannabinoids and samples positive for other drugs, implying that no crossreactivity exists between these compounds. In the case of alcohol, sulfasalazine, and meprobamate, only samples positive for both THC and drug were observed. The number of samples was small, however, and no conclusion regarding potential cross-reactivity with these compounds and/or their metabolites should be made. No confirmation of the presence or absence of THC metabolites was performed on these samples.

Compounds appearing to cause no interference with the EMIT Cannabinoid Assay include methadone, cocaine, morphine, PCP, barbiturates, amphetamines, opiates, and benzodiazepines.

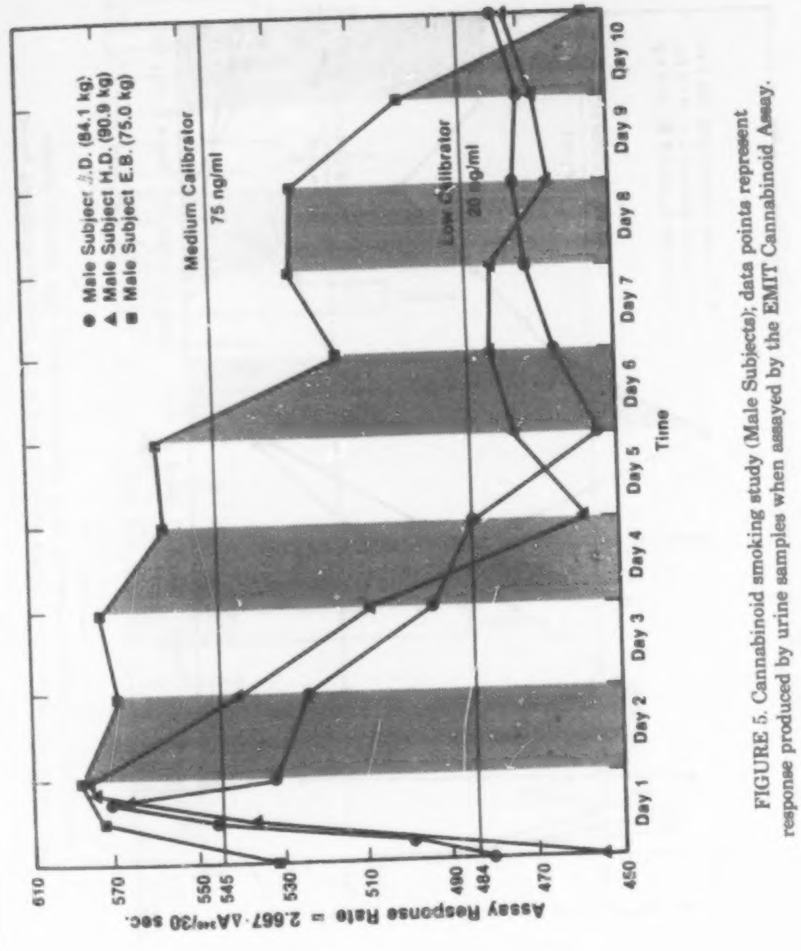


FIGURE 5. Cannabinoid smoking study (Male Subjects); data points represent response produced by urine samples when assayed by the EMIT Cannabinoid Assay.

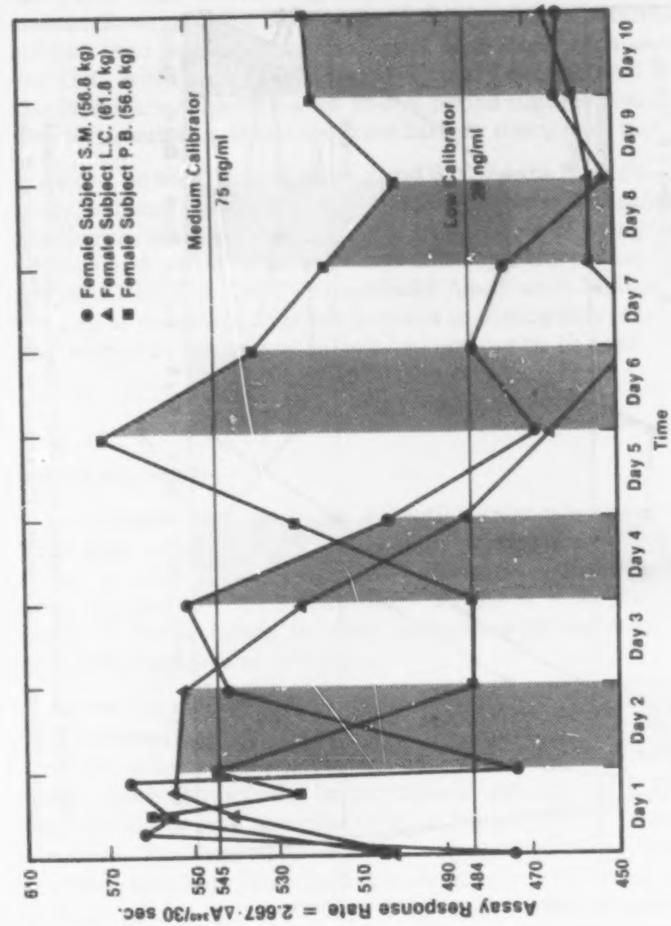


FIGURE 6. Cannabinoid smoking study (Female Subjects); data points represent response produced by urine samples when assayed by the EMIT Cannabinoid Assay.

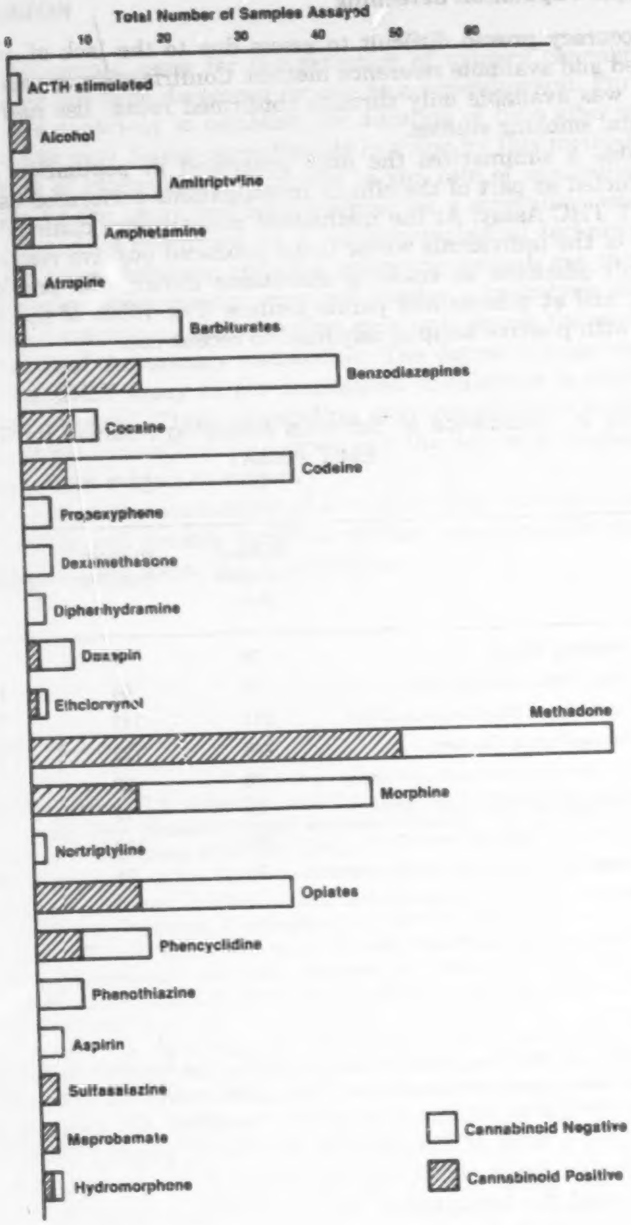


FIGURE 7. Analysis of samples known to contain drugs other than THC.

Sample Population Screening

Accuracy proved difficult to assess due to the lack of an established and available reference method. Confirmation in this evaluation was available only through confirmed recent use and experimental smoking studies.

Table 2 summarizes the data generated in population screens conducted as part of the clincal investigations performed using the EMIT THC Assay. At the methadone maintenance clinic (III), over 75% of the individuals whose urine produced positive results (>50 ng/ml) admitted to smoking marijuana during the previous 3-6 days, and at prisons and parole centers (IV), 100% of the individuals with positive samples admitted to recent use.

TABLE 2. Incidence of Samples Assaying Positive for THC by EMIT ASSAY

		Number Samples Run	Number Positive	% Positives
I.	Smoking Study	28	7*	—
II.	Drug Treatment Program	95	64	67
III.	Methadone Maintenance Clinic	211	115	55
IV.	Prison/Parole Centers	246	125*	51
V.	Toxicology/Forensic Laboratory	78	38	49
VI.	Methadone Treatment Clinic	48	12	25
VII.	Forensic/Criminal Laboratory	184	112	61
VIII.	Hospital Emergency Room Patients	96	24	25

*100% Admission to Recent Use

Within the various populations studied, the incidence of samples positive for cannabinoids ranged from 25% to 67%. The cumulative results for all populations screened identified 491 positive specimens from a total of 958 samples, an overall positive incidence of 51%. During the study, it was reported that the investigators generally found the frequency of positive results to be realistic for the populations studied.

DISCUSSION

A rapid, simple assay for the detection of urinary cannabinoid metabolites has been developed for use as a screening test. Further studies are underway to establish the duration of time after smoking that one may detect cannabinoids in urine by this method and the effects of intersubject variability in the rate of metabolism of the drug. As the assay has been designed as a screening test, any results should be confirmed by a nonimmunological technique. It should be noted, however, that the reference method, gas chromatography-mass spectrometry, may not employ a hydrolysis step in the sample extraction, and therefore may only detect the unconjugated forms of the urinary metabolites. The degree of cross-reactivity of the EMIT assay to the conjugated metabolites is currently being investigated. These metabolites may constitute a large proportion of the cannabinoids in urine and the degree of conjugation may vary from subject to subject.

It is felt that the availability of a rapid, simple screening test for cannabinoids will greatly facilitate studies regarding the medical, demographic, and social use of marijuana.

REFERENCES

1. K.E. Rubenstein, R.S. Schneider, and E.F. Ullman. Homogeneous enzyme immunoassay. New immunochemical technique.*Biochemistry and Biophysics Research Communications*, *47*,846-851 (1972).
2. H.M. Greenwood and R.S. Schneider. Current developments in the application of enzyme immunoassay. In *Clinical Immunochemistry*, S. Natilson, ed. American Assoc. Clinical Chemistry, Washington, D.C., 455-473 (1978).
3. D. Kabakoff and H.M. Greenwood. Recent developments in homogeneous enzyme immunoassay. In *Recent Advances in Clinical Biochemistry*, Vol. 2, K.G.M.M. Alberti and C.P. Price, eds., Churchill Livingstone, Edinburgh, Scotland, 1-30 (1981).
4. G.L. Rowley, T.A. Armstrong, C.P. Crowl, et al. Determination of THC and its metabolites by EMIT homogeneous enzyme immunoassay: a summary report. In *Cannabinoid Assays in Humans*, National Institute on Drug Abuse Research Monograph 7, R.E. Willette, ed. DHEW Publication No. (ADM)78-339, formerly DHEW Pub. No. (ADM)76-339, Supt. of Docs., U.S. Govt. Print. Off., Washington, D.C., pp. 28-32 (1977-78).
5. G.L. Rowley, K.E. Rubenstein, J. Huisjen, and E.F. Ullman. Mechanism by which antibodies inhibit hapten-malate dehydrogenase conjugates. *J. Biol. Chem.*, *250*,. 3759 (1975).

ACKNOWLEDGMENTS

The authors are indebted to R. Bastiani for collecting the clinical data and to Wendy Thole for her technical assistance.

AUTHORS

M. J. DeLaurentis, K. McNeil, A. J. Mann,
S. Clark, and Helena M. Greenwood
Syva Research Institute
3181 Porter Drive
Palo Alto, Calif. 94304

Analysis of Cannabinoids
Research Monograph 42, R. Hawks, Editor
National Institute on Drug Abuse, 1982

Laboratory Evaluation of Immunoassay Kits for the Detection of Cannabinoids in Biological Fluids

Michael A. Peat, Bryan S. Finkle, and Mary E. Deyman

The Center for Human Toxicology has, together with other toxicology laboratories, been involved in the field evaluation of a radioimmunoassay procedure for the detection and quantitation of Δ^9-THC in serum and an enzyme multiplied immunoassay procedure for the detection of the nor-carboxylic acid metabolite in urine. Each laboratory was selected on the basis of proven experience with commercially available immunoassay procedures. Initially, the study was designed to screen samples collected from accident casualties; however, at some of the study sites the data base was extended to include other samples.

The radioimmunoassay procedure evaluated was developed at the Receptor Research Institute, and involves the use of a tritium-labeled Δ^9-THC tracer. A total of eight laboratories participated in the evaluation of this method, which is prepared in kit form by the manufacturer. Thirteen laboratories evaluated the EMIT procedure developed at Syva Corporation.

The study protocol required that each site screen serum and urine samples and forward all presumptive positive specimens to the Center for Human Toxicology. These positive specimens were then shipped to either Battelle Laboratories or Research Triangle Institute for confirmation analysis by gas chromatography-mass spectrometry.

The results of these evaluations will be presented and will include:

1. *Correlations of positive RIA and EMIT analysis with gas chromatography-mass spectrometry results;*
2. *The results of quality-control tests;*

3. *Criticisms and suggestions made by analysts at each site;*
4. *The results of evaluations performed at the Center for Human Toxicology.*

INTRODUCTION

The widespread use of marijuana among the population of the United States has led to the need for intensive research into the pharmacological and psychological effects of Δ^9-tetra-hydrocannabinol (Δ^9-THC), the principal active ingredient in marijuana. Furthermore, Δ^9-THC is now being studied for possible therapeutic use. Together with the need to analyze plasma samples from these basic research programs there is an increasing demand from forensic and clinical toxicologists for improved analytical procedures to screen and quantitate Δ^9-THC and its metabolites in biological fluids.

One analytical technique which appears to be particularly favorable because of its sensitivity and specificity is radioimmunoassay (RIA). In fact, several groups (1,2,3,4,5) have developed such procedures. Those developed by Cook et al. (1,2) and Gross et al. (3) are reported to be specific for Δ^9-THC. The procedure developed by Gross et al. at the Receptor Research Institute (RRI) was evaluated under service laboratory conditions.

A variety of other immunoassay techniques are in common use, including the homogeneous enzyme immunoassay (EMIT) procedure. This analytical method has been applied to urine samples for the analysis of several drugs of abuse and to the quantitation of a variety of drugs and metabolites in serum. Rodgers et al. (6) developed a semiquantitative EMIT assay for cannabinoid metabolites in urine and this procedure has since been further refined. It was also evaluated during the course of the project.

The RIA kit from RRI was designed specifically for the assay of serum samples. It is a direct procedure involving no prior extraction or chromatography and uses a tritium-labeled Δ^9-THC tracer. The method uses 10 μl of serum which is incubated with 100 μl of Δ^9-THC antibody and 500 μl of the [^{3}H] Δ^9-THC tracer. Separation of the unbound Δ^9-THC is by dextran-coated charcoal, and the technique is reported to be sensitive to 5 ng/ml.

The EMIT assay was designed specifically for the detection of urinary metabolites structurally similar to 11-nor-Δ^9-THC-9-carboxylic acid (THC-9-acid). 11-Nor-Δ^9-THC-9-carboxylic acid is used to calibrate the assay. 50 μl of urine are mixed with THC antibody reagent (malate dehydrogenase covalently linked to THC) and nicotinamide adenine dinucleotide, a substrate for the enzyme. The enzymatic reaction rate is measured spectrophotometrically at 340 nm for a 30-second time period at 30°C. The procedure can detect 50 ng/ml of THC-9-acid in urine with greater than 95% confidence.

This project was designed to evaluate both the RIA and EMIT procedures under service laboratory conditions. Each laboratory in the project was primarily an analytical toxicology laboratory involved in forensic and/or clinical toxicology. The study was designed initially to screen samples collected from accident casualties and, therefore, each laboratory was requested to assure the confidentiality of the victim before inclusion in the study. During the course of the study it became increasingly apparent that the anticipated sample population would not be obtained. This was particularly so with the RIA procedure, mainly because of the number of hemolyzed blood samples encountered. In order to increase the sample population, a few laboratories were asked to use the analysis kits on other types of specimens; for example, routine drug-suspected emergency room admissions.

The study protocol required that each site screen serum and urine samples and forward all presumptive positive specimens to the Center. They were then shipped to either Battelle Laboratories or Research Triangle Institute for quantitative analysis by gas chromatography-mass spectrometry (GC-MS).

SELECTIO[illegible] OF SITES

Each laboratory in the study was selected on the basis of proven experience with commercially available immunoassay procedures. This was achieved by personal communication and by evaluation of responses to a questionnaire.

Twenty laboratories were asked originally to participate in the study. In addition, during the course of the study four further sites asked to be included and a number of sites withdrew. Seven laboratories evaluated the RIA kits and twelve the EMIT procedure. In addition to these sites, the laboratory at the Center for Human Toxicology was involved in the evaluation of both procedures.

RECEIPT AND SHIPMENT OF KITS

Evaluation of the RIA procedure began in December 1978 and continued until August 1979. Two hundred kits were received at CHT from RRI, of which 98 were used in the study. The dates of receipt and kit expiration are shown in table 1.

TABLE 1. Date of Receipt and Expiration of RIA kits

Number of Kits Received	Lot Number	Date of Receipt	Date of Expiration
10	X4A	10-10-78	1-15-79
40	X4A	11-29-78	1-15-79
50	X5A	12-20-78	2-15-79
50	X6A	4-04-79	5-15-79
50	X7A	6-26-79	8-15-79

Some laboratories, including CHT, encountered abnormally high nonspecific binding with the kits (Lot no. X6A) received on 4-4-79. Replacement antibody was forwarded to CHT on 6-26-79. All of these kits were shipped to CHT from RRI by overnight air courier service and were received in a satisfactory condition.

A total of 120 EMIT kits were received during the period May through August 1979. Although these kits were not in their final marketing form, i.e., the enzyme preparation was not lyophylized, inhouse evaluation indicated that they were satisfactory. Towards the end of the study period a small number of lyophylized kits were received and forwarded to some sites. Unfortunately, this was too late for evaluation of these particular kits to be completed.

Approximately 60 EMIT kits were shipped by overnight service to the sites or used at CHT. It is impossible to give a more accurate estimate because towards the end of the project, calibrator solutions and buffer solutions were shipped separately.

COLLECTION OF SAMPLES

The exact protocol for collection of samples at each site was the responsibility of the investigator at the particular site. It was stressed to those sites evaluating the RIA procedure that the kit was designed for the assay of plasma/serum samples and not for hemolyzed blood specimens. A number of sites, however, did in fact use the kit to screen hemolyzed blood. After collection of serum/plasma samples it was recommended that they be stored frozen in glass tubes and, if possible, protected from light. Similar recommendations were made for storage of urine samples.

best copy available

It was intended at first to limit the sample population to serum/plasma or urine collected from accident victims; however, analytical problems with hemolyzed blood samples and the late arrival of the EMIT kits made it clear that the anticipated sample population would not be met. To increase the number of specimens analyzed, it was decided to allow certain sites, including CHT, to screen other samples using the test kits. Although this increased the number of samples, the total number analyzed by RIA over a period of 12 months was still below that anticipated originally. A more statistically useful number of samples were screened in 6 months, using the EMIT procedure.

TASKS OF THE PARTICIPATING LABORATORIES

Each participant was responsible for:

1. Collection of samples
2. Analysis of samples by immunoassay
3. Shipment of presumptive positive samples to CHT by overnight air courier service

Participants were also asked to complete a questionnaire designed to obtain information about the accident and possible drug involvement, and to record the analytical results. The completed questionnaires were returned to CHT together with the presumptive positive samples.

Although these tasks appear to be relatively simple, it is important to realize that each of the participants was responsible for a forensic or clinical toxicology laboratory with a heavy routine workload. In the case of the RIA procedure, the entire assay could employ a skilled technician for as much as 2 days at a time, and this also contributed to the small number of samples screened by this technique.

CONFIRMATION OF PRESUMPTIVE POSITIVES BY GAS CHROMATOGRAPHY-MASS SPECTROMETRY

All the presumptive positives were shipped from each site to CHT by overnight air courier service They were then sent to either Dr. R. L. Foltz, of Battelle Laboratories, or Dr. J. Bursey, of Research Triangle Institute, for GC-MS confirmation. Dr. Foltz was responsible for analyzing approximately half of the plasma samples and Dr. Bursey for the remainder of these samples and all of the urine samples.

The analytical method used to assay the plasma samples was designed to detect Δ^9-THC specifically, and that used for the urine samples to detect the 11-nor-Δ^9-carboxylic acid metabolite. A small number of urine specimens were hydrolyzed before extraction.

QUALITY CONTROL SAMPLES

At regular intervals throughout the study, serum samples "spiked" with Δ^9-THC were supplied on a blind basis to each site evaluating the RIA procedure. These were prepared according to the following protocol: A 203 mg/ml Δ^9-THC solution supplied by NIDA was serially diluted to a 20.3 (A) and 2.03 ng/μl (B) concentration with ethanol. These solutions were then used to "spike" serum; for example, 50 μl of B in 25 mls of serum resulted in a Δ^9-THC concentration of 4.06 ng/ml. Each batch of samples was shipped in dry ice by overnight air courier service to each laboratory.

RESULTS

RIA

The participants evaluating the RIA procedure analyzed a total of 310 samples of which 43 (14 percent) were identified as presumptive positives. Approximately one-third of this latter group were hemolyzed. The results of quantitation of samples analyzed by both RIA and GC-MS are shown in table 2. The number of hemolyzed samples included in this group is unknown.

Quality control specimens, prepared in serum, were sent to each site participating in the RIA evaluation. Table 3 shows the results of these analyses. Some of the regression coefficients were as follows:

GC-MS against RIA:	Total minus sample 4	(n = 97)	r = 0.777
"Spiked concentration" against:	GC-MS (samples 1-22*)	(n = 21)	r = 0.959
	CHT (samples 1-22*)	(n = 29)	r = 0.802
	RRI (samples 1-12*)	(n = 19)	r = 0.950

* Excluding sample 4

In addition to analyzing the quality control specimens, a number of plasma samples from the Emergency Room of the University of Utah Medical Center were also analyzed at CHT; these were paired

TABLE 2. Results of RIA and GC-CIMS Analysis

Site	RIA (ng/ml)	GC-MS (ng/ml)	Site	RIA	GC-MS (ng/ml)
I	8.6	0	IV	Positive	86
	5.0	Not Analyzed		Positive	31
	6.4	0		Positive	220
	6.5	Less than 1		Positive	155
	15	9.3		Positive	72
	9.0	9.9		Positive	69
	23	Interference		Positive	11
	8.5	6.5		Positive	3.0
	9.4	0		Positive	21
	6.5	6.2		Positive	0
	15	5.0		Positive	10
II	7.0	2.0		Positive	0
III	15	10.5		Positive	5.2
	9.0	3.0		Positive	4.6
	4.0	0		Positive	3.1
				Positive	Interference

with urine samples collected from the same patient and screened positive by EMIT. Table 4 shows the data obtained by RIA and GC-MS.

EMIT

A total of 13 laboratories, including CHT, were involved in the evaluation of this procedure during the period June to December 1979. The 12 sites screened a total of 596 samples from accident victims, of which 165 (28%) were identified as presumptive positives.

Some of these sites used the EMIT procedure to screen for positives and negatives only, whereas others determined the concentration of metabolites present. It must be remembered that initially the GC-MS procedure quantitated only the free 11-nor-carboxylic acid metabolite, and Williams and Moffatt (7), reported that up to 80% of the acid metabolites are conjugated in the urine. From these initial data, 18 of the samples were screened positive by EMIT but were negative for the carboxylic acid metabolite by GC-MS. Subsequently several samples with low or negative GC-MS results were hydrolyzed enzymatically and then reanalyzed by GC-MS for the 11-nor-9-carboxylic acid metabolite. Table 5 shows the results of these analyses.

TABLE 3. Results of Quality-Control Specimens (All Concentrations ng/ml)

Quality Control Sample	Date	Spiked Value	CHT	I	II	III	IV	V	VI	VII	GC-MS Analysis*
1	Jan 79	0	6.0	5.5	7.0	5.8	8.0, 8.4				0
2	Jan 79	4.1	11.0	Sample^C broke	11.0	8.5	10.5, 11.0				4.0
3	Jan 79	20.3	18.0	38.6	23.0	20.0	27.0, 26.0				19.3
4	Jan 79	81^C	130^C	170^C	110^C	>50^C	58^C, 82^C				73.4^C
5^A	May 79	0^C	3, 5	<2, <2	0	0, 0		0			0
6^B	May 79	15	24, 28	15, 12	20.5	14.7, 17.1		20			20.4
7	May 79	0^C	8, 8	<2, <2	0	0, 0		4.8			0
8	May 79	25	41, 46	25, 25	30.5	25.3, 28.4		30			29.6
9	May 79	5	18, 19	5, 5	11.4	6.9, 11.1		12.5			9.1
10^B	May 79	15	31, 31	15, 16	20.3	11.7, 17.1		18			16.9
11	May 79	0^C	9, 12	<2, <2	4.3	0, 0		3			0
12^A	May 79	0^C	3, 6	<2, <2	0	0, 0		0			0
13^B	Jul 79	6	11						7.5	14	10.1
14^B	Jul 79	6	10						7.5	11	10.6
15^A	Jul 79	0^C	0						0	0	0.7
16	Jul 79	2	4						2.8	5.6	4.1
17^A	Jul 79	0^C	0						0	0	0.7
18	Jul 79	10	11						13	NA	14.3
19	Jul 79	22	26						31	NA	26.4
20^B	Jul 79	14	17						18	NA	15.7
21	Jul 79	18	24						25	NA	21.9
22^B	Jul 79	14	17						15	NA	13.2

A-Blank Plasma samples

B-Split pairs of samples (numbers 6 and 10, 13 and 14, 20 and 22)

C-Not included in regression analysis

* Battelle Laboratories

TABLE 4. Plasma Samples from Subjects Whose Urine was Screened Positive by EMIT

Sample No.	RIA (ng/ml)	GC/MS (ng/ml)
1	7	0
2	0	0
3	<5	0
4	<5	0
5	11	0
6	0	1.3
7	0	0
8	10	<1
9	0	2.1
10	0	0

TABLE 5. Results of EMIT and GC-MS Analyses After Enzymatic Hydrolysis

EMIT (ng/ml)	GC-MS of Free Acid (ng/ml)	GC-MS After Hydrolysis (ng/ml)
Positive	4.6	42
Positive	0	4
Greater than 75	36	600
53	7.1	32
0	0	4
Greater than 75	31	75
10	6.5	7.9
11	0	2.7
75	6.2	58
0	24	Less than 2
0	0	47
Greater than 75	5	31
Greater than 75	2.1	53
Greater than 75	0	43
Greater than 75	2.1	77

CHT was closely involved in the evaluation of the EMIT procedure and examined a large number of samples. Of these, 106 were analyzed by both EMIT and GC-MS. The results are tabulated in table 6. A total of 37 samples screened positive by EMIT but were unconfirmed by GC-MS for the free acid metabolite. Subsequently 3 of those were hydrolyzed enzymatically and found to be positive by

TABLE 6. Results of EMIT Analysis at CHT and GC-MS Analysis for the Free Acid Metabolite at RTI

EMIT (ng/ml)	GC-MS (ng/ml)	EMIT (ng/ml)	GC-MS (ng/ml)	EMIT (ng/ml)	GC-MS (ng/ml)
12	0	0	0	57	20
30	4.0	0	0	Greater than 75	50
Greater than 75	21	12	11	15	7.5
14	8.1	Greater than 75	36	58	22
Greater than 75	55	0	0	36	2.6
Greater than 75	9.1	53	7.1	60	30
34	26	23	0	Greater than 75	26
37	35	Greater than 75	31	27	3.3
15	0	10	6.5	Greater than 75	41
21	0	11	0	Greater than 75	7
0	0	0	0	60	9
18	17	Greater than 75	6.2	Greater than 75	45
0	0	0	24	Greater than 75	11
55	24	0	2.3	Greater than 75	5
15	4.4	19	0	Greater than 75	2.1
20	20	10	17	54	0
16	0	35	0	Greater than 75	2.1
11	2.2	15	4	33	145
0	0	0	29	10	4
62	29	0	20	24	0
0	0	Greater than 75	10	Greater than 75	2.1
66	38	10	4	22	2.5
15	5	74	22	56	78
0	20	24	25	Greater than 75	37
0	0	50	2.5	Greater than 75	39
14	26	0	10.5	58	70
12	0	Greater than 75	3.4	37	18
14	14	0	0	Greater than 75	67
0	0	0	7.8	33	2.5
Greater than 75	5	0	7	Greater than 75	27
30	0	13	5	25	26
21	3.5	72	32	Greater than 75	0
57	2.9	35	4	34	0
52	3.6	25	0	40	2.6
37	0	23	0	18	0
52	42				

GC-MS. More importantly, perhaps, 8 samples from the CHT data screened negative by EMIT but positive by GC-MS, although 4 of these had free carboxylic acid metabolite concentrations of less than 15 ng/ml (i.e. less than the low calibrator for EMIT) by GC-MS. It should be noted that the 12 participants did not forward any "negatives" for GC-MS confirmation, so the true number of "false negatives" may have been higher.

Plasma and urine samples from some patients were analyzed by EMIT, RIA and GC-MS methods. Of those plasma samples in which no Δ^9-THC was detectable by GC-MS, 12 of the corresponding urines were positive by both EMIT and GC-MS, 5 plasmas positive by GC-MS, 11 of the corresponding urines were positive by both EMIT and GC-MS, whereas 3 were positive by EMIT only, although in these cases the urines were not hydrolyzed.

DISCUSSION

Certainly the most disappointing aspect of the entire study was the lack of response to the RIA evaluation. The time-consuming procedure and problems with the kit itself were the most common explanations given by the participants. In addition, the kit was designed to assay serum samples, whereas the real need among those laboratories chosen for the study was for a kit to screen hemolyzed blood samples and, in fact, the kit was inappropriately used at some laboratories to do this.

The correlation between GC-MS and RIA on the case samples analyzed by the participants was far from adequate. At the 6 sites reporting data, six false positives (1.9% of the total, 13.9% of the presumptive positives) were found, although it is possible that some of these were hemolyzed. Of 10 plasma samples analyzed at CHT from subjects with positive urines (by EMIT), 2 showed values by RIA above the sensitivity limit of the assay which were subsequently shown to be zero by GC-MS.

Six of the laboratories and CHT were involved in the analysis of the quality control samples. The data from these samples is shown in table 3. It is interesting to note that the performance at CHT improved throughout the study as the laboratory staff became more familiar with the procedure. It is also worthwhile to note that those sites with personnel experienced in RIA techniques performed better than those new to the use of tritiated tracers.

The regression coefficients (GC-MS vs. RIA) for participating sites are greater than 0.93 for all but two sites. One of these achieved only 0.46, but this is much improved if one obvious error is eliminated. Since the kit is prepared in a form suitable for marketing, consideration must be given to the fact that laboratory experience with this type of kit is essential for adequate results.

At the end of the study a questionnaire was forwarded to each site. One of the questions asked was at what lower concentration limit the kit was unreliable when applied to serum. There was, of course, variation in the responses, one laboratory suggesting 2 ng/ml, three suggesting 5 ng/ml and two 10 ng/ml. Our own experience at CHT suggests a limit between 5 and 10 ng/ml when the procedure is used by inexperienced personnel. Whether this is adequate for forensic case work is questionable in view of recent data on the blood concentrations of Δ^9-THC to be expected in medicolegal cases (Williams and Moffatt, 1980). The kit instructions indicate an expected sensitivity of 5 ng/ml when applied to serum.

Each participant was also asked to describe briefly his or her views concerning the kit and the instructions and to comment on the usefulness of the procedure. Generally, all the participants found the kit and instructions to be satisfactory. Most, however, reported that they had trouble with the standards at the lower end of the curve (5 to 10 ng/ml), particularly with reproducibility from assay to assay, and they suggested that samples falling in this range be repeated. One participant reported:

> "Overall, I feel that the kit should be developed and placed into laboratory use, especially in cases involving traffic accidents, drunken driving, bizarre accidents and coroner's cases in general."

This view was echoed by the other participants.

A total of 12 laboratories and CHT evaluated the EMIT procedure. Because the technique was simple and familiar, all sites found the kit easy to use. The kit was compared to a GC-MS assay developed by Dr. Joan Bursey, of Research Triangle Institute. The assay was designed to quantitate the 11-nor-Δ^9-THC-9-carboxylic acid metabolite, whereas the kit was designed to detect "cannabinoid metabolites" which resembled this acid metabolite in structure. This fact alone could explain the poor correlation between the EMIT values and those obtained by GC-MS, particularly those samples with considerably higher EMIT values. However, if the data in table 4 are considered, it is much more likely that the discrepancy is due to the EMIT responding to the glucuronide conjugate of this metabolite. Unfortunately, in most cases the sample volume was insufficient for reanalysis of the urine by GC-MS after hydrolysis.

It is much more difficult, however, to account for those samples with a significantly lower EMIT value:

EMIT (ng/ml)	GC-MS (ng/ml)
0	20
14	26
0	29
0	20
0	10.5
0	7.8
0	7
33	145

As with the RIA procedure, a questionnaire was sent to each participant, requesting views and comments. Generally, the participants had few problems. Some questions were raised concerning the procedure for running blanks and whether the kit was satisfactory for postmortem urine because of the extreme turbidity sometimes seen in such samples. The introduction of the lyophilized reagents has been reported to alleviate these problems.

The interpretation of EMIT urine analyses from both a medicolegal and clinical viewpoint should be approached with caution. Of 34 cases in which both plasma and urine were analyzed, both samples were positive in only 14 cases, showing that a positive EMIT reading does not necessarily correlate with the recent smoking of marijuana, if the presence of Δ^9-THC in plasma is taken to indicate recent use. In addition, a practical difficulty is that the confirmation of the urine EMIT readings is not a straightforward process because current GC-MS procedures are designed to quantitate only the free 11-nor-Δ^9-THC-9-carboxylic acid metabolite. Hydrolysis of the conjugated metabolites becomes important if such techniques are to be used.

CONCLUSIONS

There is a demand for analytical screening procedures for Δ^9-THC in serum and/or blood and for cannabinoid metabolites in urine. Development of the EMIT procedure has come a long way towards satisfying the latter demand, although some effort should be directed towards developing a more complete confirmatory procedure.

The analysis of Δ^9-THC in serum and/or blood is more complex. In this case the GC-MS confirmatory method is acceptable, although further refinements are needed so that it will be applicable to all types of blood samples and so metabolites can be clearly separated and identified.

Screening serum and blood samples using a tritiated RIA kit is still a very difficult and time-consuming procedure. The kit should not be used by inexperienced personnel for quantitation of Δ^9-THC.

To help overcome some of the problems encountered with the RIA procedure evaluated, we would recommend the development of an iodinated (I^{125}) tracer for Δ^9-THC and also for either the 11-hydroxy or carboxylic acid metabolite. In our opinion, the latter would improve both the quantitation and the confidence of the screening assay.

REFERENCES

1. C.E. Cook, M.L. Hawes, E.W. Amerson, et al. *Pharmacologist, 18,* 291 (1976).
2. C.E. Cook. ACS Symposium Series 98, Am. Chem. Soc., Washington, D.C., 137-154 (1979).
3. S.J. Gross, J.R. Soares, S.L. Wong, et al. *Nature, 252,* 581 (1974).
4. J.D. Teale, E. Forman, L.J. King, et al. *Nature, 249,* 154 (1974).
5. A.R. Chase, P.R. Kelley, A. Taunton-Rigby, R.T. Jones, and T. Harwood. Quantitation of cannabinoids in biological fluids by radioimmunoassay. In R.E. Willette, ed. National Institute on Drug Abuse Research Monograph 7,*Cannabinoid Assays in Humans.* DHEW Pub. No. (ADM)76-339. Supt. of Docs., U.S. Govt. Print. Off., Washington, D.C., 1-9 (1976).
6. R. Rodgers et al. *Clin. Chem. 24,* 95 (1978).
7. P.L. Williams and A.C. Moffat. *J. Pharm. Pharmac., 32,* 445 (1980).

AUTHORS

Michael A. Peat, Bryan S. Finkle, and Mary E. Deyman
Center for Human Toxicology
University of Utah
Salt Lake City, Utah 84112

Analysis of Cannabinoids
Research Monograph 42, R. Hawks, Editor
National Institute on Drug Abuse, 1982

Quantitative Analysis for Δ^9-THC, 11-Hydroxy-Δ^9-THC, and 9-Carboxy-Δ^9-THC in Plasma Using GC/CI-MS

Rodger L. Foltz and Bruce J. Hidy

A method for quantitative measurement of Δ^9-THC concentrations in plasma has been developed and applied to the analysis of more than 2000 samples over the past 4 years. The method includes addition of deuterium-labeled Δ^9-THC to the plasma for use as the internal standard, a simple and relatively rapid solvent extraction procedure, formation of the trimethylsilyl derivative, and quantitation by selected ion monitoring using ammonia chemical ionization. A similar procedure has also been developed for simultaneous analysis of Δ^9-THC and two of its major metabolites in plasma. The latter procedure requires use of a glass capillary column for assays of plasma samples in which the Δ^9-THC concentration is below 10 ng/ml. Sensitivities for both methods permit quantitation of THC and its metabolites at concentrations as low as 0.2 ng in 1-ml plasma samples.

INTRODUCTION

Interest in the pharmacology of cannabinoids continues at a high level, owing to the widespread use of marijuana and the discovery of potentially beneficial medical applications for Δ^9-tetrahydrocannabinol. Cannabinoid research has been hampered by the limited availability of analytical facilities capable of measuring the typically low levels of cannabinoids in physiological fluids after cannabinoid administration. For the past 4 years Battelle Columbus Laboratories has provided an analytical service to NIDA-authorized scientists engaged in cannabinoid research. The cannabinoid assays developed and used at Battelle are based on the combination of gas chromatography and chemical ionization mass spectrometry (GC/CI-MS).

QUANTITATION OF Δ^9-THC

Measurement of Δ^9-THC in plasma by a GC/MS method was first reported by Agurell et al. (1). Their procedure includes addition of a deuterium-labeled THC analog to the plasma, extraction, and liquid chromatographic purification on a Sephadex LH-20 column, followed by quantitative measurement using gas chromatography and electron impact mass spectrometry (GC/EI-MS). The method used at Battelle is similar except that it uses a relatively simple extraction based on the procedure (figure 1) developed at the Research Triangle Institute (2), and employs ammonia chemical ionization rather than electron impact ionization (3). Using this procedure, a set of 36 plasma samples can be extracted and prepared for analysis by one technician in a period of 8 hours. An additional 8 hours is required to perform the GC/MS analyses.

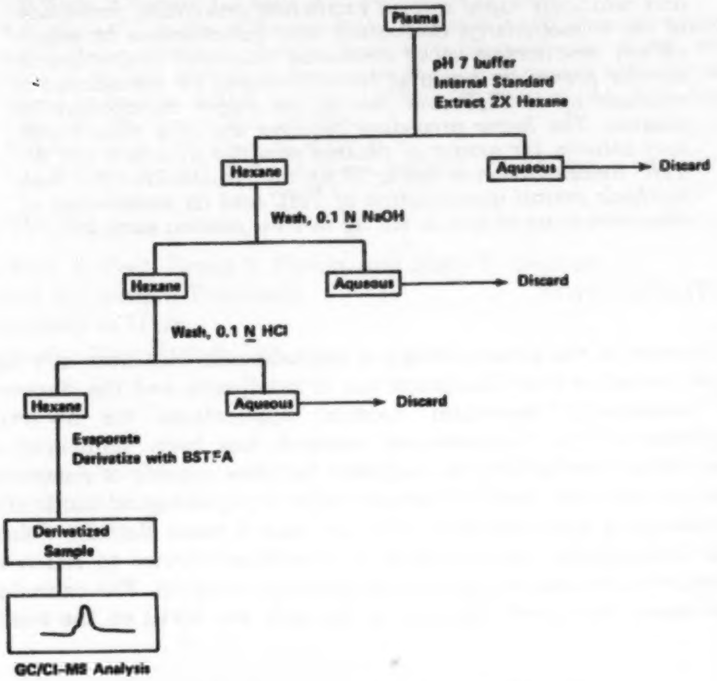

Plasma
pH 7 buffer
Internal Standard
Extract 2X Hexane
Hexane
Aqueous
Discard
Wash, 0.1 N NaOH
Hexane
Aqueous
Discard
Wash, 0.1 N HCl
Hexane
Aqueous
Discard
Evaporate
Derivatize with BSTFA
Derivatized Sample
GC/CI-MS Analysis

FIGURE 1. Flow diagram for analysis of Δ^9-THC in plasma.

Most of the THC analyses done at Battelle during the past 2 years have been performed on a Finnigan 4000 GC/MS coupled to an Incos data system. With this system operating normally, as little as 20 pg of derivatized THC injected into the GC/MS gives a response sufficient to permit measurement with good precision. Usually, only 10 to 20 percent of a derivatized extract is injected at one time. Consequently, for a 1-ml plasma sample, concentrations of THC as low as 0.2 ng/ml can be measured.

EXTRACTION PROCEDURE

All glassware should be thoroughly cleaned and silylated (4). The THC standard solutions should be stored at −10°C in the dark but allowed to warm to room temperature just prior to use.

Transfer a measured quantity (normally 1 ml) of plasma to a 20-ml culture tube equipped with a Teflon-lined screw cap. Add 100 µl of an ethanol solution of the Δ^9-THC-$^2H_3$ internal standard (200 ng/ml) followed by 1.0 ml of a pH 7.0 buffer solution (Fisher Scientific Co.). Vortex the mixture for 10 sec. Add 6 ml of high purity hexane (Distilled-in-Glass, Burdick and Jackson Co.), tightly cap the tube, and gently mix the contents for 20 min using an automated tube rocker or rotator. Centrifuge for 6 min and then carefully transfer the hexane (top) layer to a second culture tube using a disposable Pasteur pipette. The remaining plasma residue can be discarded.

The hexane solution is now washed successively with dilute alkali and dilute acid to remove strongly acidic and basic compounds. Add 2.5 ml of 0.1 N NaOH solution to the hexane extract. After capping the tube, rotate or rock for 20 min and then centrifuge. Using another disposable pipette, remove the aqueous (bottom) layer and discard. Add 2.5 ml of 0.1 N HCl solution to the hexane extract. Tap the tube gently to cause gas bubbles formed by the acid-base reaction to escape. Again, tightly cap the tube, rock or rotate it for 10 min, and centrifuge. Carefully transfer just the hexane (top) layer to a 15-ml glass tube with a tapered or cone-shaped bottom. Gently concentrate the extract to near dryness at a temperature of 35-40°C using either a nitrogen stream or a rapid evaporation device such as the Buchler Vortex-evaporator (Buchler Instruments).

The extract residue is now transferred to a 1-ml glass vial having a cone-shaped bottom and equipped with a Teflon-lined screw cap in the following manner. Add 0.5 ml of high purity pentane to the tube containing the residue and vortex the tube for 10 sec. Using a disposable pipette, transfer the pentane solution to the 1-ml glass vial. Repeat the last step using an additional 0.5 ml of pentane to transfer any remaining extract residue to the 1-ml vial. Add 10 μl of high purity dimethylformamide to the pentane solution to act as a "keeper" to minimize evaporative loss of the THC during the final evaporation and to serve as a solvent for the trimethylsilation. Slowly concentrate the pentane solution by heating the glass vial with a heating block maintained at 40°C. When all of the pentane has evaporated (approximately 20 min), add 15 μl of bis-(trimethylsilyl) trifluoroacetamide containing 1 percent trimethylsilyl chloride to the vial and cap tightly. Vortex the vial and then heat in an oven at 90°C for at least 1 hour. The sample is now ready for GC/MS analysis. However, the sample can be stored at −10°C indefinitely.

GC/MS ANALYSIS

Chemical ionization with ammonia as the reagent gas is used for quantitation of the trimethylsilylated THC because it affords higher sensitivity and specificity than either electron impact ionization or chemical ionization using methane or isobutane as reagent gases (5). The method was developed on a quadrupole system, but any mass spectrometer equipped for chemical ionization and selected ion monitoring should be satisfactory, provided it has adequate sensitivity. For systems in which the gas chromatograph is connected directly to the mass spectrometer, methane (Ultrahigh Purity grade, Matheson Gas Products, East Rutherford, NJ) should be used as the carrier gas. If the GC/MS employs a separator, helium may be used as the carrier gas. In both cases the ammonia (Ultrahigh Purity grade, Air Products and Chemicals, Tamaqua, PA) should be introduced into ion source via a make-up gas inlet so that the ammonia and carrier gases enter the ion chamber in concentric gas streams. The ammonia flow rate into the ion source is adjusted while observing ion intensities at m/z 18 and 35. As the ammonia flow rate is increased, the intensity of the m/z 18 peak (NH_4^+) should at first increase and then reach a plateau, at which point a peak at m/z 35 ($NH_3 \cdot NH_4^+$) appears and begins to increase in intensity. The optimum ammonia flow rate is the point at which the m/z 18 peak reaches a maximum and the m/z 35 peak just begins to increase in height. The total reagent gas pressure in the ion chamber should be between 0.3 and 1.0 torr. If a separator is used, it may be necessary to bleed in methane along with the ammonia to achieve this total pressure.

Various gas chromatographic columns can be used, but the best results have been achieved with a 1.8-m × 2-mm ID glass column packed with 3 percent OV-17 on 100/120 mesh Gas Chrom Q (Applied Science Labs, State College, PA). The injector, column, and transfer-line temperatures are maintained at 280°, 250°, and 320°C, respectively. Under these conditions and a carrier gas flow rate of approximately 15 ml/min, the Δ^9-THC-TMS should elute in approximately 4 min. The resolution of the GC column can be evaluated by injecting a solution containing an equal mixture of Δ^8-THC-TMS and Δ^9-THC-TMS (≅50 μg/ml in hexane). Under the stated GC conditions a good quality OV-17 column will give a chromatogram showing at least a detectable valley between the peaks corresponding to the two cannabinoid derivatives.

For selected ion monitoring the mass spectrometer is set up to monitor the ion currents at m/z 387 and 390, corresponding to the protonated-molecule ions (MH+) for Δ^9-THC-TMS and Δ^9-THC-$^2H_3$-TMS, respectively. After the GC/MS run is completed, the Δ^9-THC plasma concentration is determined by measuring the areas of the Δ^9-THC-TMS and Δ^9-THC-$^2H_3$-TMS peaks in the selected ion current profiles ("ion chromatograms") and relating the peak areas ratio to a previously established standard curve. A new standard curve should be established whenever new standard solutions are prepared or when analysis of plasma samples containing known concentrations of Δ^9-THC shows unacceptable accuracies. For routine analysis of plasmas containing unknown concentrations of THC, at least 1 out of every 6 samples should be a spiked standard.

Figure 2 shows a typical selected ion current profile with peaks corresponding to 5 ng/ml of Δ^9-THC-TMS and 20 ng/ml of Δ^9-THC-$^2H_3$-TMS, while figure 3 is a standard curve for the THC concentration range 1 to 100 ng/ml of plasma. Precision data for various concentrations of THC in plasma are listed in table 1.

EXTRACTION OF HEMOLYZED BLOOD

The described procedure has proven to be a reliable method for the analysis of THC in plasma and normal whole blood samples. However, attempts to analyze hemolyzed and badly decomposed blood samples gave erratic results due to poor extraction efficiencies. Both the THC originally in the blood and the added deuterium-labeled THC apparently are tightly bound to something in the

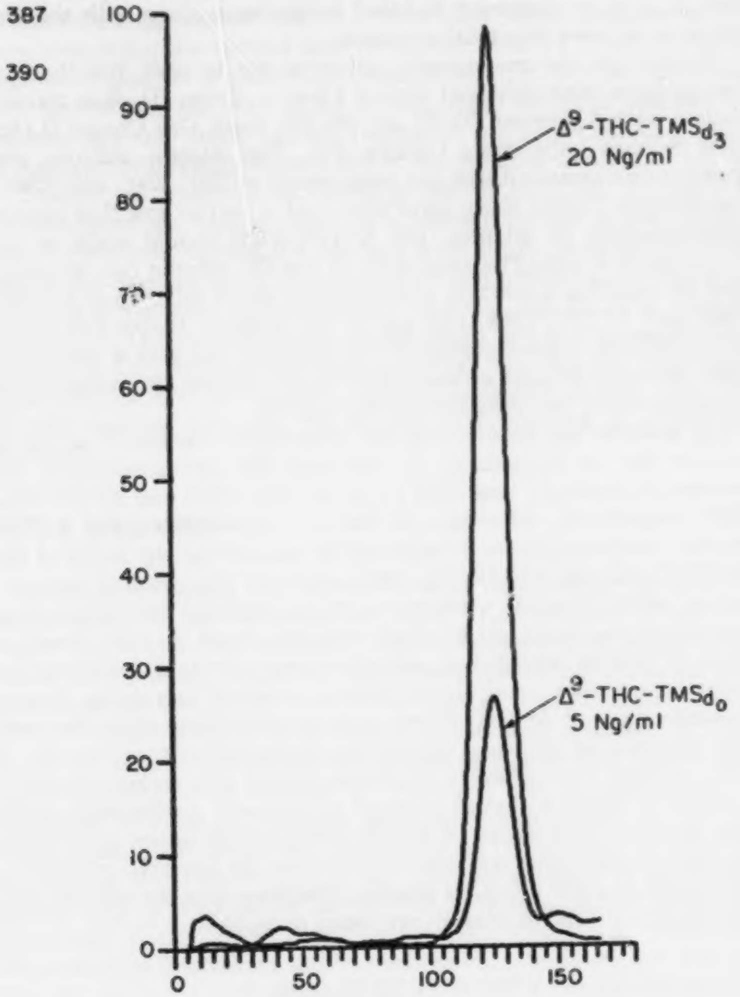


FIGURE 2. Selected ion current profiles for quantitation of Δ^9-THC in plasma.

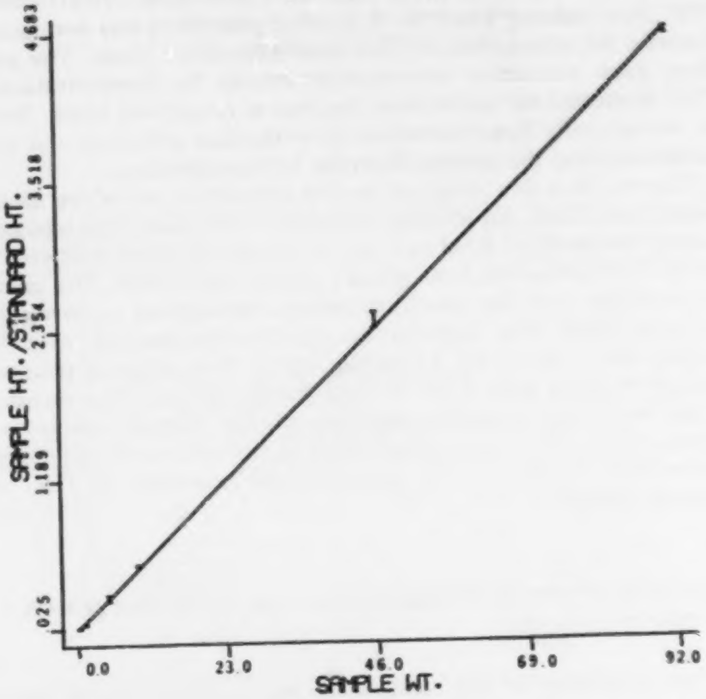


FIGURE 3. Standard curve for measurement of Δ^9-THC in 1-ml plasma samples.

TABLE 1. Precision Data for Measurement of Δ^9-THC Added to Plasma*

Δ^9-THC Added (ng/ml)	Relative Standard Deviation (%)
92	0.5
46	3.0
9.2	3.9
4.6	5.1
0.9	7.3

*4 samples analyzed twice at each concentration.

hemolyzed blood, thereby preventing efficient extraction with hexane. Rosenthal and Brine reported a procedure for extraction of THC from cadaver blood (6). A simpler procedure was developed at Battelle for extraction of THC from hemolyzed blood. The procedure gave acceptable quantitative results for concentrations of THC down to 1 ng/ml for most batches of hemolyzed blood. However, occasionally large variations in extraction efficiency did occur, indicating that the method deserves further attention.

Figure 4 is a flow diagram for the extraction procedure used for hemolyzed blood. An ethanol solution of the deuterium-labeled internal standard is added to 1 ml of hemolyzed blood followed by 1 ml of 3 N H_2SO_4 and 1 ml of high purity acetonitrile. The mixture is sonicated and the resulting protein precipitate removed after centrifugation. The supernatant is then neutralized with 3 N NaOH and 1 ml of pH 7.0 buffer added. The aqueous solution is extracted twice with 6 ml of high purity hexane. The remaining steps involving successive washing of the hexane extract with dilute alkali and dilute acid followed by concentration and derivatization are identical to the procedure for extraction of THC from normal plasma.

QUANTITATION OF 9-CARBOXY-Δ^9-THC AND 11-HYDROXY-Δ^9-THC IN PLASMA

The 11-hydroxy-Δ^9-THC metabolite has psychotomimetic activity comparable to that of Δ^9-THC. Consequently, any study attempting to relate behavior effects to THC blood concentrations should also include analysis of the 11-hydroxy metabolite blood concentrations. Although the 9-carboxy metabolite has no psychomimetic activity, it is a major THC metabolite, and its concentration in both blood and urine can be measured for a longer period of time than the concentration of Δ^9-THC. Consequently, there has been strong interest in developing methods for quantitation of these two metabolites in blood and other body fluids. A method capable of simultaneously measuring plasma concentrations of THC and the two metabolites would be particularly attractive.

Rosenfeld has published a method for simultaneous quantitation of Δ^9-THC and the 11-hydroxy-Δ^9-THC in plasma involving extraction into toluene followed by back extraction of phenolic compounds into Claisen's alkali and derivatization prior to GC/MS analysis (7).

A method for determination of Δ^9-THC, cannabinol, and 11-hydroxy-Δ^9-THC has been developed at the Research Triangle Insti-

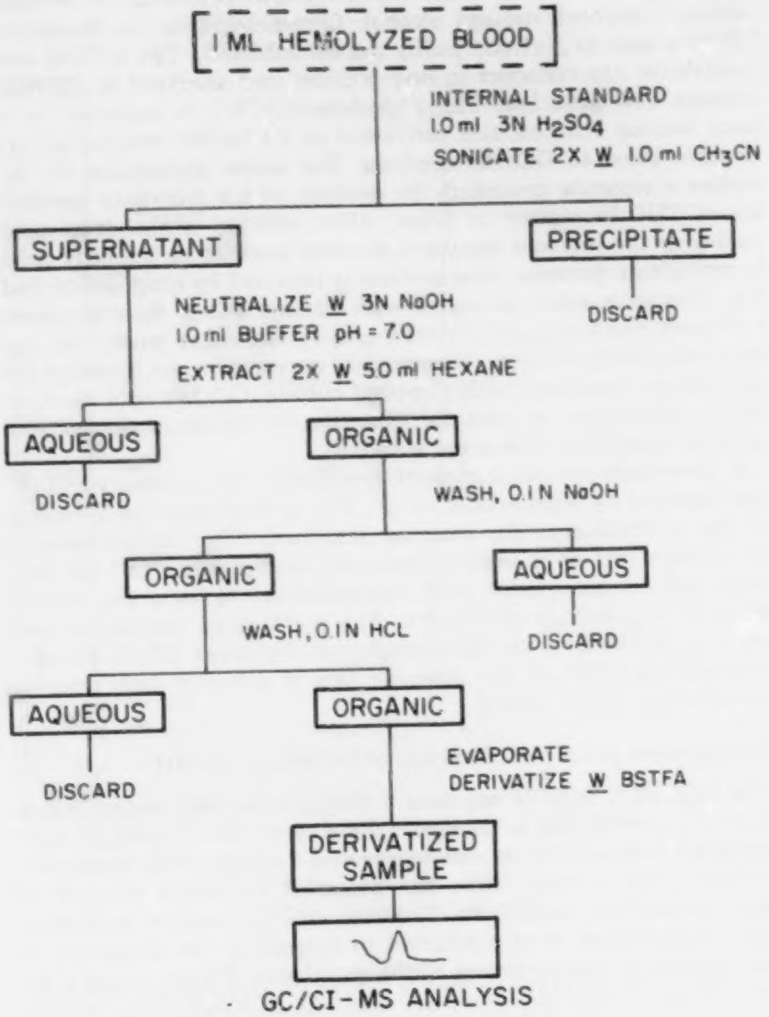

I ML HEMOLYZED BLOOD
INTERNAL STANDARD
1.0 ml 3N H_2SO_4
SONICATE 2X W 1.0 ml CH_3CN
SUPERNATANT
PRECIPITATE
NEUTRALIZE W 3N NaOH
1.0 ml BUFFER pH = 7.0
EXTRACT 2X W 5.0 ml HEXANE
DISCARD
AQUEOUS
ORGANIC
DISCARD
WASH, 0.1 N NaOH
ORGANIC
AQUEOUS
WASH, 0.1 N HCL
DISCARD
AQUEOUS
ORGANIC
DISCARD
EVAPORATE
DERIVATIZE W BSTFA
DERIVATIZED SAMPLE
GC/CI-MS ANALYSIS

FIGURE 4. Flow diagram for analysis of Δ^9-THC in hemolyzed blood.

tute (8). After addition of deuterated internal standards to the plasma, the cannabinoids are extracted into petroleum ether containing 1 percent isoamyl alcohol. Chromatography on Sephadex LH-20 is used to partially purify the cannabinoids. The Δ^9-THC and cannabinol are collected in one fraction and analyzed by GC/MS without derivatization. The 11-hydroxy-Δ^9-THC is collected in a later eluting fraction and converted to its bis-(trimethylsilyl) derivative prior to GC/MS analysis. The same publication (8) describes a separate procedure for analysis of the 9-carboxy metabolite of THC in plasma or urine. After addition of the deuterated analog as the internal standard, acetone is added to the body fluid to precipitate proteins. The acetone is removed by evaporation and the aqueous residue extracted with diethyl ether. Reverse phase high performance liquid chromatography is used to purify the carboxy metabolite which is subsequently converted to its dimethyl derivative by treatment with dimethyl sulfate. GC/MS with electron impact ionization is used for quantitative determination of the ratio of metabolite to internal standard.

A procedure for measurement specifically for 9-carboxy-Δ^9-THC was reported by Nordquist et al. (9). The method uses the Δ^9-isomer of the metabolite as the internal standard. After acidification of the plasma (pH 4), it is extracted with diethyl ether and the concentrated extract treated with diazomethane to form the methyl ester of the carboxy metabolite. The methylated extract is then partially purified by chromatography on Sephadex LH-20. Finally, trimethylsilylation of the phenolic OH is achieved just prior to quantitation by GC/EI-MS.

Investigation of LC/MS Analysis of 9-Carboxy-Δ^9-THC

In May 1978 Battelle acquired a Finnigan LC/MS system which utilizes a moving belt to transport the eluent from a liquid chromatographic column into the ion source of a Finnigan 4000 mass spectrometer. The system offers the potential for direct analysis of polar metabolites such as the 9-carboxy-Δ^9-THC without derivatization. Experiments were conducted to determine the sensitivity of this system for measurement of the metabolite. Figure 5 shows the ion current profile obtained by depositing on the moving belt, by means of a syringe, a hexane solution containing 1 ng of the 9-carboxy-Δ^9-THC. Unfortunately, this sensitivity could not be approached when the metabolite was passed through a reverse phase HPLC column and then onto the moving belt. The major causes for dramatically reduced sensitivity appeared to be increased background due to the solvent system and a longer period of elution. Because of the relatively poor sensitivity of the LC/MS for this compound, attention was shifted to development of a GC/MS method.

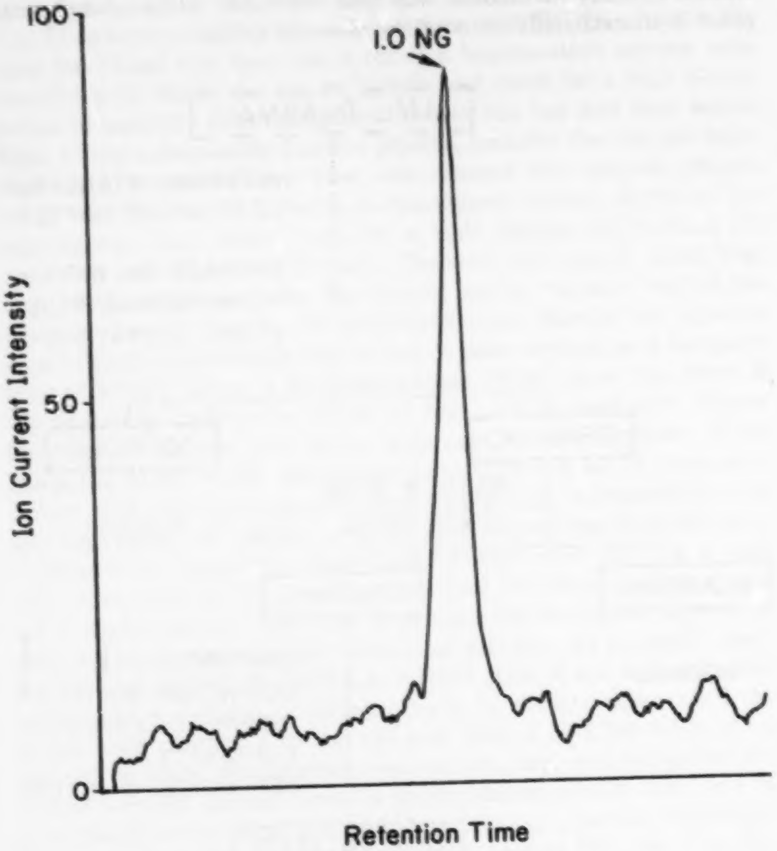


FIGURE 5. Ion current profile for MH^+ of 9-carboxy-Δ^9-THC introduced into the mass spectrometer via the LC/MS interface.

GC/MS Method

A procedure was sought for simultaneously extracting Δ^9-THC and the two metabolites of interest from plasma. This was achieved

by modifications of the previously described procedure for extraction of Δ^9-THC from plasma. The major changes consisted of use of a more polar solvent and elimination of the dilute alkali wash. Also, the best GC/MS reproducibility was experienced when the extracted carboxy metabolite was first converted to its methyl ester prior to trimethylsilylation of the phenolic group.

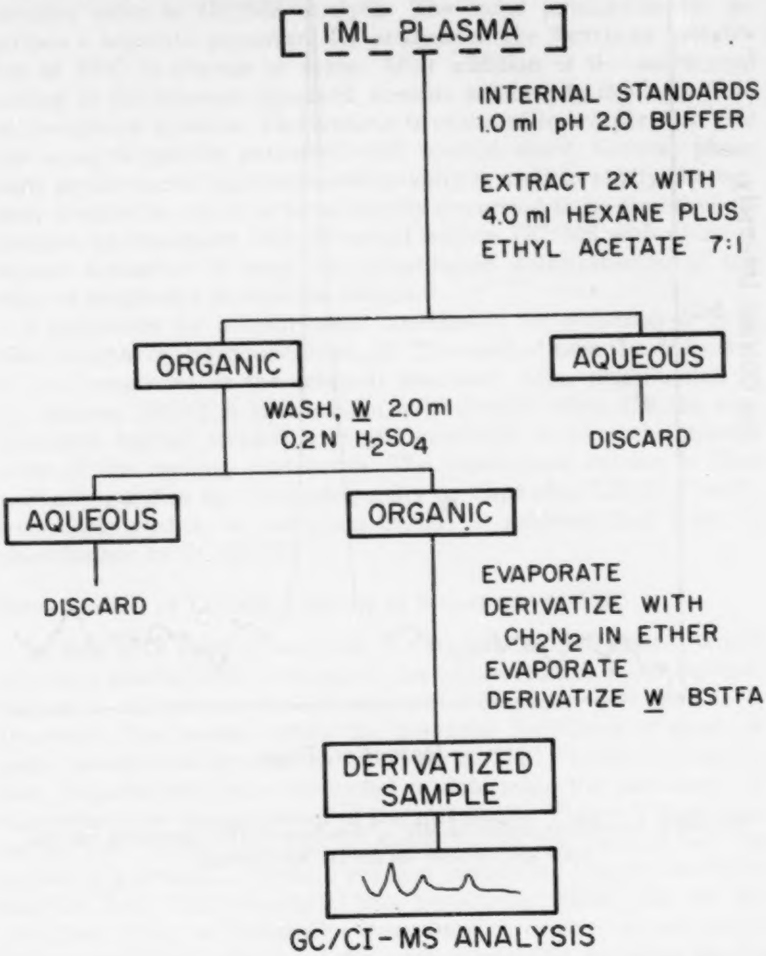

1ML PLASMA
INTERNAL STANDARDS
1.0 ml pH 2.0 BUFFER
EXTRACT 2X WITH
4.0 ml HEXANE PLUS
ETHYL ACETATE 7:1
ORGANIC
AQUEOUS
WASH, W 2.0 ml
0.2 N H_2SO_4
DISCARD
AQUEOUS
ORGANIC
DISCARD
EVAPORATE
DERIVATIZE WITH
CH_2N_2 IN ETHER
EVAPORATE
DERIVATIZE W BSTFA
DERIVATIZED SAMPLE
GC/CI-MS ANALYSIS

FIGURE 6. Flow diagram for simultaneous analysis of Δ^9-THC, 11-hydroxy-Δ^9-THC, and 9-carboxy-Δ^9-THC in plasma.

Figure 6 is a flow diagram for the extraction of Δ^9-THC and its two metabolites of interest from plasma. To 1 ml of plasma in a 20-ml culture tube equipped with a Teflon-lined screw cap, add 1 ml of a pH 2 buffer, followed by 100 μl of an ethanol solution containing 50 ng each of the deuterium-labeled internal standards (Δ^9-THC-$^2H_3$, 11-hydroxy-Δ^9-THC-$^2H_3$, and 9-carboxy-Δ^9-THC-$^2H_3$). Vortex the tube for 10 sec and then add 4 ml of a hexane-ethyl acetate solution (7:1 v/v). Screw the cap on tightly and check for a leak. Slowly rotate or rock the tube for 15 min. Loosen the cap and then centrifuge. Using a disposable Pasteur pipette, transfer the organic layer (top) to a second culture tube and discard the aqueous plasma layer. Add 2 ml of 0.2 N H_2SO_4 to the organic extract. Screw on the cap, tighten, and again check for a leak. Repeat the rocking (10 min) and centrifugation (10 min). Transfer the organic layer (top) to a 15-ml centrifuge tube. Be careful not to transfer any of the aqueous (lower) layer to the centrifuge tube. Discard the aqueous layer. Gently concentrate the extract to near dryness at a temperature of 35-40°C using a nitrogen stream. Wash down the sides of the centrifuge tube with 0.5 ml of high purity methanol. Vortex the tube for 10 sec and again evaporate to near dryness. Wash down the sides of the centrifuge tube with 0.2 ml of methanol. Vortex the tube for 10 sec and then transfer the methanol solution to a 1-ml glass vial having a cone-shaped bottom and equipped with a Teflon-lined screw cap. High purity diethyl ether (0.5 ml) is used for a final wash of the centrifuge tube and the ether combined with the methanol in the 1-ml vial. Evaporate the combined solvents to near dryness using a gentle stream of nitrogen while gently heating the vial (≈40°C). To form the methyl ester of the carboxy metabolite, add a solution of diazomethane in diethyl ether until the yellow color persists. Cap the vial and heat at 50°C for 0.5 hr. Cool the vial to room temperature, remove the cap, and evaporate the solvent to near dryness under a gentle stream of nitrogen. Add 50 μl of bis-(trimethylsilyl) acetamide containing 1 percent trimethylsilyl chloride to the vial, cap tightly and heat at 90°C for 1 hr. The sample is now ready for GC/MS analysis, or it may be stored in a freezer (−10°C) until it is to be analyzed.

For GC/MS analysis a 1.8-m × 2-mm glass column packed with 3 percent OV-17 on 100/120 mesh Gas Chrom Q (Applied Science Labs, State College, PA) can be used. However, at THC concentrations below about 10 ng/ml, interference by an endogenous, acidic component of plasma prevents accurate measurement of the THC

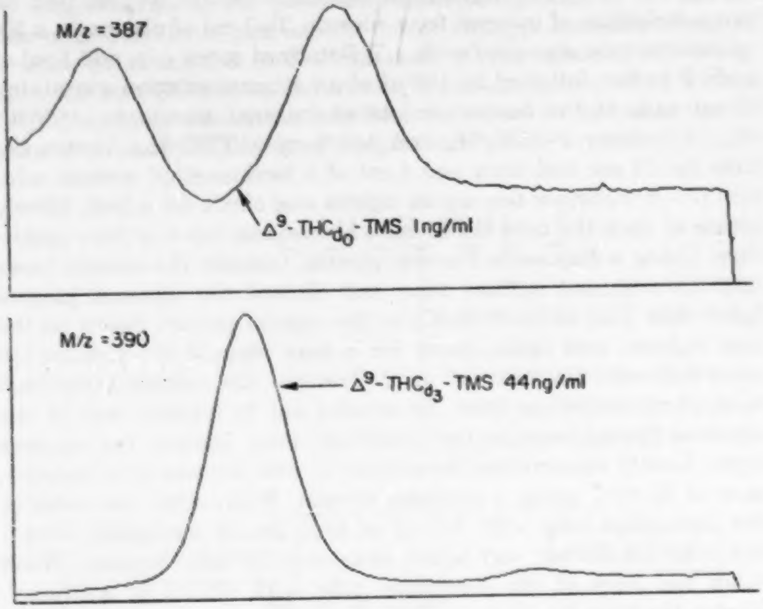


FIGURE 7. Selected ion current profiles for packed column GC/MS analysis of Δ^9-THC.

peak height (see figure 7). The interfering peak can be resolved from the THC peak by use of a capillary column.

Figure 8 shows the selected ion current profiles corresponding to the protonated-molecule ions for Δ^9-THC-TMS (m/z 387) and Δ^9-THC-$^2H_3$-TMS (m/z 390) from an analysis conducted on a 15-m glass capillary (0.25 mm I.D.) coated with SE-52.

In this example, the Δ^9-THC was present at a concentration of only 1 ng/ml, and it is well resolved from other peaks in the chromatogram, in contrast to the packed column selected ion current profile (figure 7) where the peak, due to the Δ^9-THC-TMS, is completely obscured by the interfering peak. The capillary column assay involves a splitless injection at a column temperature of 160°C of 2 μl of the silylated extract plus 1 μl of n-tetradecane which gives enhanced GC resolution due to the so-called "solvent effect" (10). After 1 min the column temperature is rapidly programmed to 250°C. Under these conditions all three of the cannabinoids are eluted within 6 min as shown in figure 9.

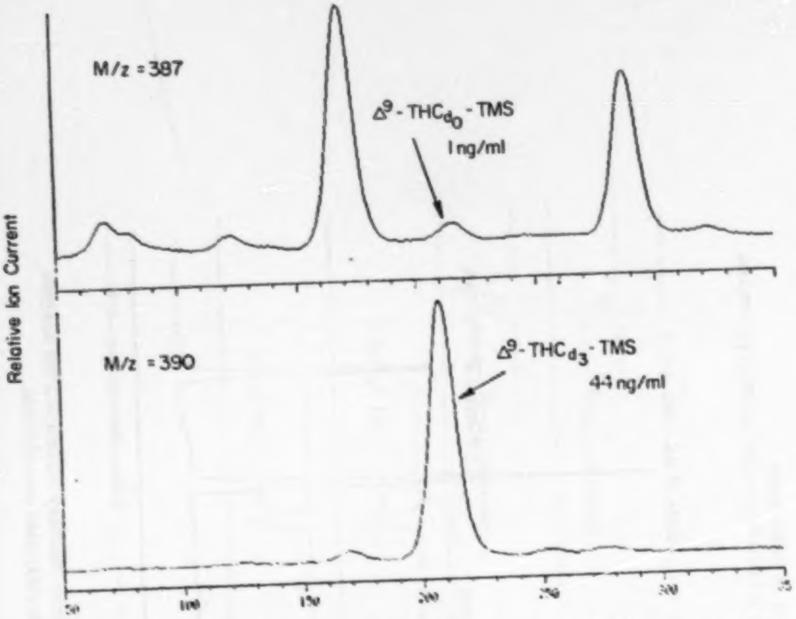


FIGURE 8. Selected ion current profiles for capillary column GC/MS analysis of Δ^9-THC.

In order to achieve maximum sensitivity, only two ion masses are monitored at a given time during the GC/MS analysis. Thus, the ion masses corresponding to the protonated-molecule ions for Δ^9-THC-TMS and its deuterated analog are monitored during their elution window. Then, under computer control, the mass analyzer voltages are switched so that the ions corresponding to the protonated-molecule ions for the 11-hydroxy-Δ^9-THC-TMS and its deuterated analog are monitored. Finally, the mass analyzer voltages are set to monitor the protonated-molecule ion currents for the methyl ester of the 9-carboxy-Δ^9-THC-TMS and its deuterated analog. A computer printout of the resulting ion current profiles is shown in figure 10.

Improved capillary column GC resolution can be achieved without lengthening the analysis time by using hydrogen as the carrier gas rather than helium or methane (11). Furthermore, the combination of hydrogen and ammonia gives better quality CI mass spectra than the combination of helium and ammonia. The hydrogen/ammonia CI mass spectra for each of the trimethylsilylated cannabinoids are shown in figure 11.

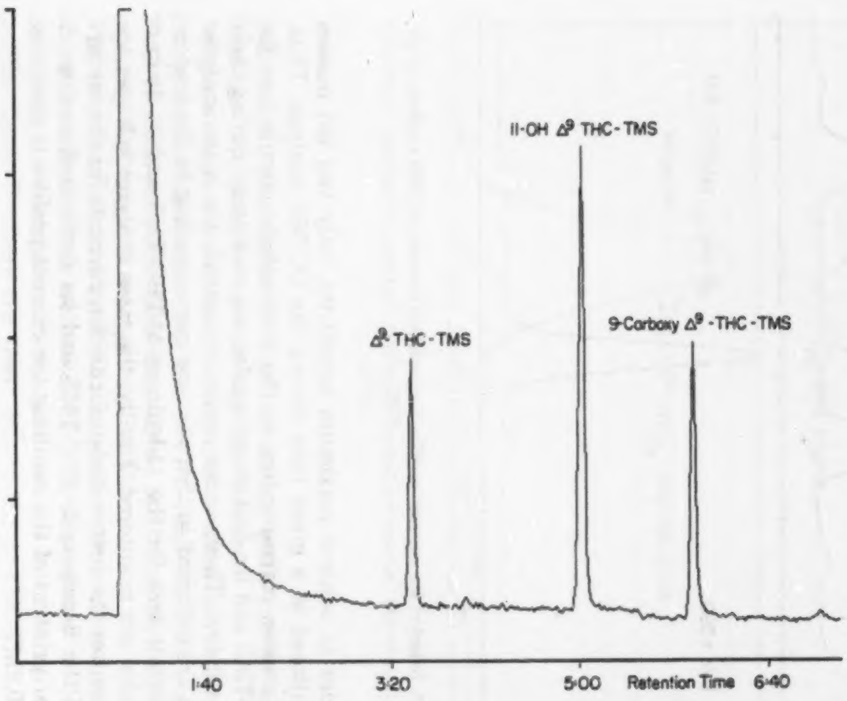


FIGURE 9. Total ion current chromatogram from capillary column GC/MS analysis of standard mixture of the trimethylsilylated cannabinoids.

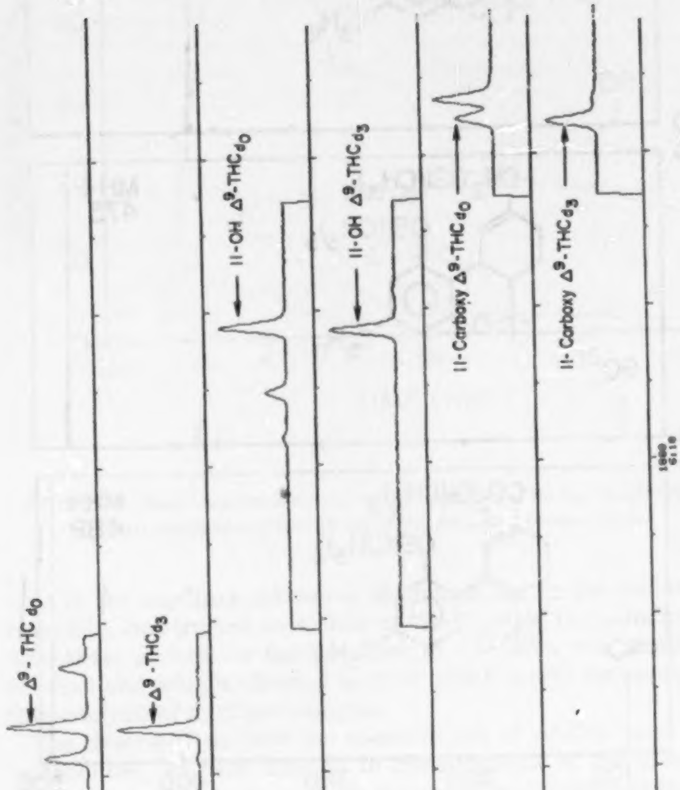


FIGURE 10. Computer printout of selected ion current profiles for GC/MS analysis of Δ9-THC and two of its metabolites.

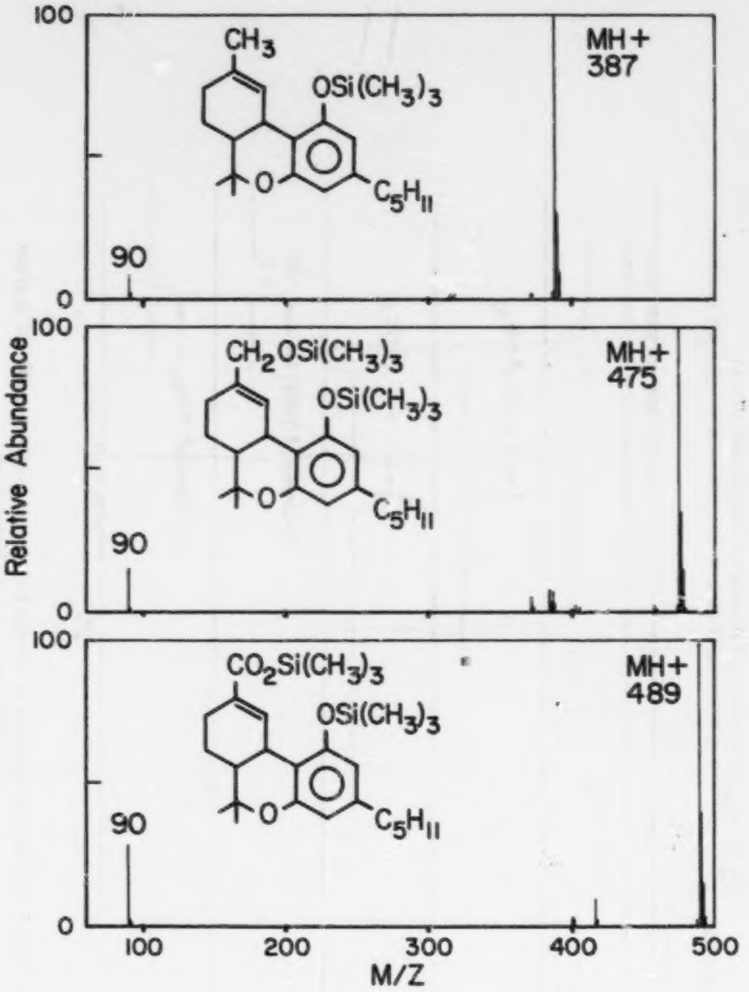


FIGURE 11. CI-MS (H_2/NH_3) of trimethylsilylated cannabinoids.

Despite the high resolving power of capillary column GC and the specificity of ammonia CI-MS, it is evident from figure 10 that interfering peaks are still a potential problem. This is largely due to the relatively unselective extraction method used. An additional negative consequence of the extraction procedure is that the life-

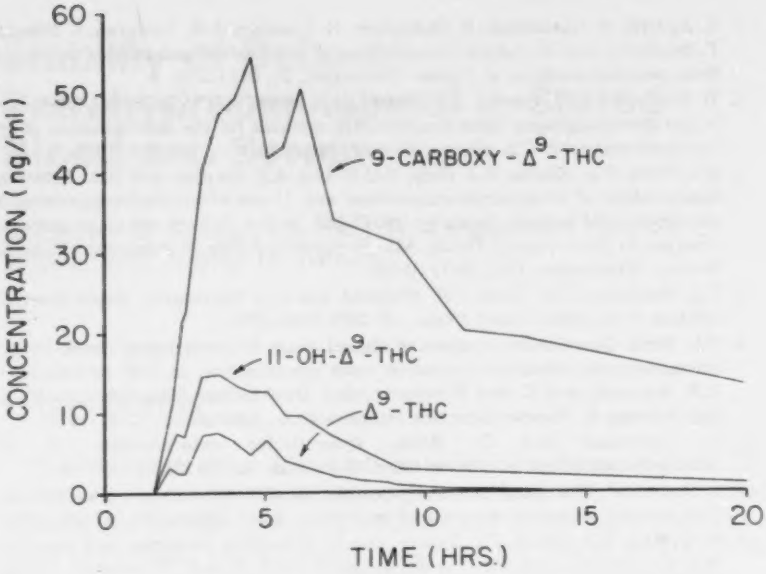


FIGURE 12. Blood concentrations of Δ^9-THC and two of its metabolites following oral administration of 10 mg of the drug to a human subject.

time of the capillary column is shortened due to the buildup of intractable, coextracted materials at the front of the column. Therefore, there is need for further work to develop a more selective extraction and sample cleanup method which is still amenable to routine analysis of multiple samples.

The described method for quantitation of Δ^9-THC and the two metabolites has been applied to measurement of the plasma concentrations of these compounds following oral administration of 10 mg of Δ^9-THC to human volunteers. Figure 12 shows the concentrations versus time curves for a representative case.

ACKNOWLEDGMENT

These studies were supported by National Institute on Drug Abuse Contract HSM-42-72-183.

REFERENCES

1. S. Agurell, B. Gustafsson, B. Holmstedt, K. Leander, J.-E. Lindgren, I. Nilsson, F. Sandbery, and M. Asberg. Quantitation of Δ^9-tetrahydrocannabinol in plasma from cannabis smokers. *J. Pharm. Pharmacol., 25*, 554 (1973).
2. D. Rosenthal, T.M. Harvey, J.T. Bursey, D.R. Brine, and M.E. Wall. Comparison of gas chromatography mass spectrometry methods for the determination of Δ^9-tetrahydrocannabinol in plasma. *Biomed. Mass Spectr., 5*, 312-316 (1978).
3. R.L. Foltz, P.A. Clarke, B.J. Hidy, D.C.K. Lin, A.P. Graffeo, and B.A. Petersen. Quantitation of Δ^9-tetrahydrocannabinol and 11-nor-Δ^9-tetrahydrocannabinol-9-carboxylic acid in body fluids by GC/CI-MS. In J.A. Vinson (ed.), *Cannabinoid Analysis in Physiological Fluids*, ACS Symposium Series 98, American Chemical Society, Washington, D.C., 59-71 (1979).
4. D.L. Fenimore, C.M. Davis, J.H. Whitford, and C.A. Harrington. Vapor-phase silylation of glassware. *Anal. Chem., 48*, 2289-2290 (1976).
5. R.L. Foltz. Quantitative analysis of abused drugs in physiological fluids by gas chromatography/chemical ionization mass spectrometry. In A.P. de Leenheer, R.R. Roncucci, and C. van Peteghem (eds.), *Quantitative Mass Spectrometry in Life Sciences II*, Elsevier Scientific Publishing Co., Amsterdam, 39-62 (1978).
6. D. Rosenthal and D. Brine. Quantitative determination of Δ^9-tetrahydrocannabinol in cadaver blood. *J. Forensic Sci., 24*, 282-290 (1979).
7. J. Rosenfeld. The simultaneous determination of Δ^9-tetrahydrocannabinol and 11-hydroxy-Δ^9-tetrahydrocannabinol in plasma. *Anal. Letters, 10*, 917-930 (1977).
8. M.E. Wall, D.R. Brine, J.T. Bursey, and D. Rosenthal. Detection and quantitation of tetrahydrocannabinol in physiological fluids. In J.A. Vinson (ed.), *Cannabinoid Analysis in Physiological Fluids*, ACS Symposium Series 98, American Chemical Society, Washington, D.C., 39-57 (1979).
9. M. Nordquist, J.-E. Lindgren, and S. Agurell. A method for the identification of acid metabolites of tetrahydrocannabinol by mass fragmentography. In R.E. Willette (ed.), *Cannabinoid Assays in Humans*, NIDA Research Monograph 7, DHEW Pub. No. (ADM)78-339. Supt. of Docs., U.S. Govt. Print. Off., Washington, D.C., 64-69 (1976).
10. K. Grob and K. Grob, Jr. Splitless injection and the solvent effect. *J. High Resolution Chromatogr. and Chromatogr. Commun., 1*, 57-64 (1978).
11. K. Grob and G. Grob. Practical capillary gas chromatography–a systematic approach. *J. High Resolution Chromatogr. and Chromatogr. Commun., 2*, 109-117 (1979).

AUTHORS

Rodger L. Foltz
Center for Human Toxicology
University of Utah
Salt Lake City, Utah 84112

Bruce J. Hidy
Battelle Memorial Institute
505 King Avenue
Columbus, Ohio 43201

Analysis of Cannabinoids
Research Monograph 42, R. Hawks, Editor
National Institute on Drug Abuse, 1982

Stability of Delta-9-Tetrahydrocannabinol in Stored Blood and Serum

Anthony S. Wong, Michael W. Orbanosky, Victor C. Reeve, and James D. Beede

INTRODUCTION

The literature has described delta-9-tetrahydrocannabinol (Δ^9-THC) as a degradable molecule, subject to dissipation when exposed to oxidation (1), elevated temperatures, and active surfaces such as glass and plastic which contribute (2-6) to the loss of Δ^9-THC from aqueous media by extensive and sometimes irretrievable binding.

Since our work deals principally with the analysis of Δ^9-THC in blood, concern developed regarding whether Δ^9-THC would exhibit similar instability in blood samples stored over relatively long periods. This study was designed to evaluate long term storage when Δ^9-THC is present in blood in high physiological concentrations. We examined the dissipation rate of Δ^9-THC during storage, the effects of elevated temperatures on blood-THC samples, the effects of repetitive freeze/thaw cycles on blood-THC samples, and the effects of agitation and ambient temperature fluctuation during mailing.

EXPERIMENTAL DESIGN

All blood and serum samples in this study were spiked with Δ^9-THC at a concentration of 50 ng/ml by adding 50 ng of Δ^9-THC dissolved in 5 µl of alcohol to each ml of blood or serum. All glassware containing the concentrated standards was silylated prior to use. Glassware used to contain blood-THC samples was left untreated. The quantitative analytical procedure used in this study involved the addition of 50 μl of C-11 deuterated Δ^9-THC to 1.0 ml of blood or serum, acidification of the sample to pH 4-5 using 3 ml 0.1 M KH_2PO_4 followed by multiple 5 ml extractions with 1.5% isoamyl alcohol in pentane. The combined organic phases were evaporated just to dryness and the residue dissolved in 50 μl of 0.1 M trimethylphenyl ammonium hydroxide, of which 5 μl were injected into the GC/MS. The fundamental difference between this method and that developed by Rosenfeld (5) is the absence of a prior cleanup of the extract.

A Finnigan 3200 GC/MS interfaced with a two-channel programmable multiple ion monitor (PROMIM) was employed for the analysis. Gas chromatography was conducted on a 60 × 0.4 cm glass column containing 1.5% SP-2250/1.95% SP-2401 on 100/120 Supelcoport at 210°C. Selected ions were monitored at m/e 328 and m/e 331, the molecular ions for methylated Δ^9-THC and its deuterated analog, respectively. Figure 1 and figure 2 represent an ion chromatogram for Δ^9-THC and a typical standard curve for unextracted Δ^9-THC versus 5 ng deuterated THC.

The precision and accuracy of the analytical method in hemolyzed blood and serum were determined by spiking replicates at two concentration levels: 10 ppb (10 ng Δ^9-THC in 5 μl of ethanol to 1 ml of substrate) and 50 ppb (50 ng of Δ^9-THC in 5 μl of ethanol to 1 ml of substrate). All the replicates of the same substrate were analyzed on the same day immediately after spiking.

1. Long Term Storage: 1.0 ml samples of spiked blood or serum were prepared and stored in glass under nitrogen at 5°C, under ambient atmosphere at 5°C, -5°C, and -20°C. Storage stability was evaluated on a biweekly basis.
2. Elevated Temperature: Triplicates of 1.0 ml samples of blood and serum were stored and maintained at 20°, 30°, 40°, 50° and 60°C for 25 hours prior to analyses.
3. Repetitive Freeze/Thaw Cycles: Triplicate 1.0 ml samples of blood and serum were frozen at -20°C for 8 days during which time the samples were allowed to thaw for 4 hours each day and refrozen. Analyses were performed on the eighth day.
4. Mailing Effect: Δ^9-THC (50 μg in 5 μl of ethanol) was spiked to 1.0 ml of hemolyzed blood and serum in 2 ml glass bottles equipped with Teflon-lined screw caps. The containers were then mailed to southern California, returned to Sacramento, and the Δ^9-THC analyzed.

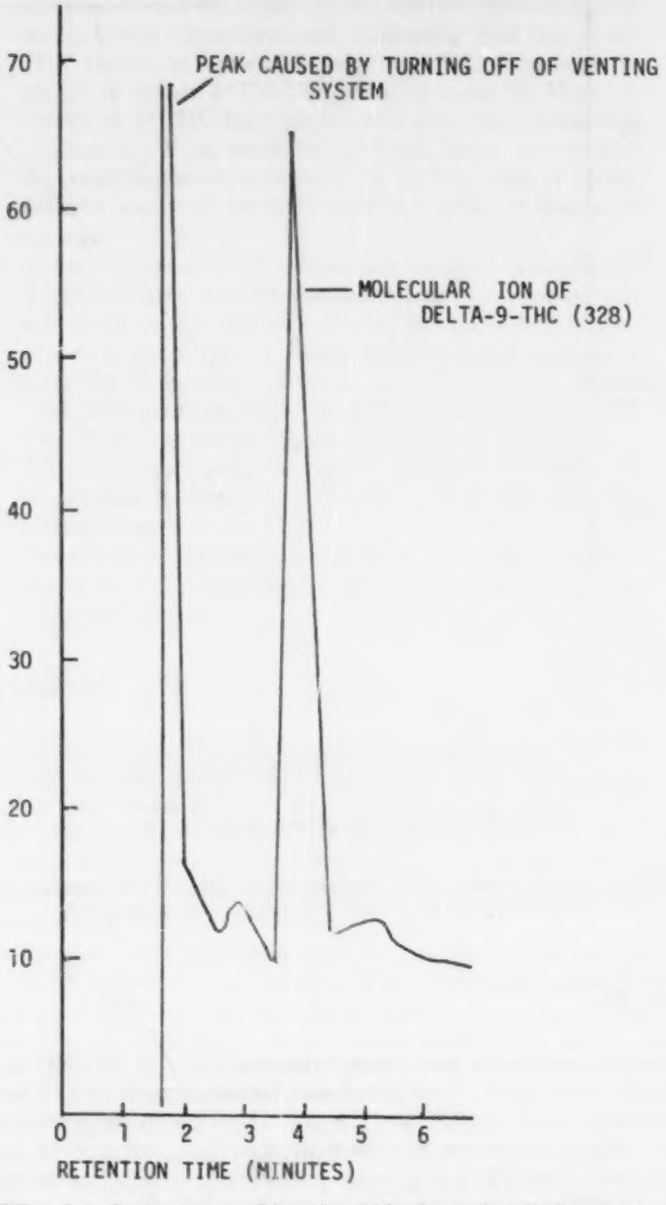


FIGURE 1. Ion chromatogram of hemolyzed blood sample spiked with 10 ppb of delta-9-THC.

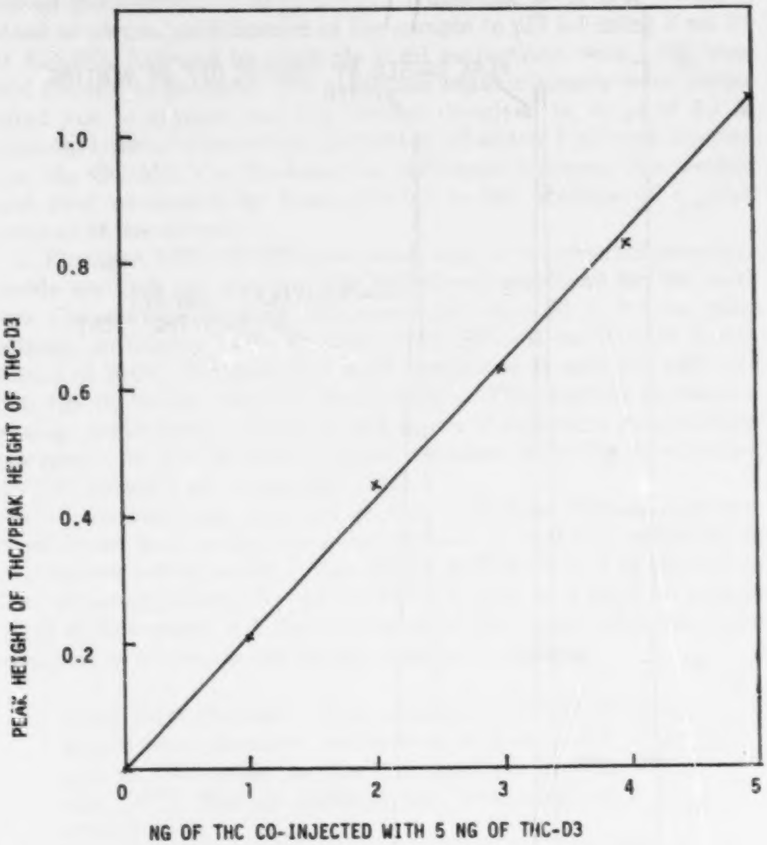


FIGURE 2. Standing curve of THC co-injected with THC-D3. The curve covers the range of 0 to 50 ng of THC in the original blood sample.

RESULTS

Relative recoveries and assay reproducibility of Δ^9-THC in ten replicate analyses of 1.0 ml blood and serum samples spiked at concentrations of 50 ng/ml and 10 ng/ml respectively were determined. Mean recoveries for blood at the 50 ng/ml and 10 ng/ml blood levels were 100% (CV=4%) and 97% (CV=5%), respectively.

1. Δ^9-THC appeared to remain stable in blood up to and including week 17. The analyses conducted on the

samples stored to weeks 19, 21, and 23 showed significantly lower concentrations, indicating that loss of Δ^9-THC begins at approximately week 19. Complete inability to detect Δ^9-THC occurred at week 23. High recovery of Δ^9-THC from serum was achieved up to week 13. Starting from week 15, however, lower and irregular analyses were obtained. As in the case of blood, definite losses of Δ^9-THC occurred after 19 weeks in storage.

2. In both blood and serum samples, neither atmospheric conditions nor storage temperatures contributed significantly to the recovery of Δ^9-THC. Delta 9-THC was found to be stable in blood samples when stored at 60°C for 25 hours. In serum, Δ^9-THC could be recovered from samples stored at 50°C for 25 hours. At 60°C the serum matrix gelled, which inhibited extraction.
3. Both blood and serum samples containing Δ^9-THC can be repeatedly frozen and thawed without showing significant losses of the drug.
4. There was no significant loss from the substrates with agitation in mailing during normal handling with the containers utilized.

DISCUSSION

The sudden losses of Δ^9-THC that occurred around the 17th week in storage cannot be sufficiently explained. It is thought, nonetheless, that the losses are not due to actual degradation or sudden surface adsorption of the Δ^9-THC molecule. Rather, it is surmised that the dissipation of Δ^9-THC may be due to the inability successfully to extract the drug, possibly owing to irretrievable binding of the molecule to degrading proteins.

The recovery data from the replicate analyses of the spiked samples clearly indicate the suitability of the analytical method for detection of Δ^9-THC in both hemolyzed blood and serum. Comparison of the ion chromatogram of a typical hemolyzed blood sample spiked with 10 ppb of Δ^9-THC (figure 1) and the standard curve (figure 2) shows that the peak of the molecular ion, 328, of methylated Δ^9-THC is about 50% of full scale with a noise level of less than 5% of full scale. The calculated detection limit of Δ^9-THC should be between 1 and 2 ppb (1 to 2 ng/ml of blood). This detection limit is comparable to that reported by other researchers (2,5,7). Since the physiological levels normally observed in marijuana users range from 1 ng/ml to 100 ng/ml in blood (1), the method developed during the present study to evaluate the *in vitro* stability of Δ^9-THC can also be used to determine the Δ^9-THC in the blood of the smokers.

The analytical technique is time-consuming and the GC/MS instrumentation is expensive. Therefore, the technique is not a practical screening procedure. The use of deuterated Δ^9-THC as an internal standard is essential in obtaining precise and accurate results.

REFERENCES

1. R.K. Razdan, A.J. Putlick, B.A. Zitko, and G.R. Hendrich. *Experientia, 28,* 121 (1972).
2. D. Rosenthal, D. Brine, and M. Wall. Quantitation of Delta-9-Tetrahydrocannibinol in Human Serum by Gas Chromatography-Mass Spectrometry. A special report presented to National Institute on Drug Abuse, October 1, 1976.
3. R. Mechoulam. *Science, 168,* 1159 (1970).
4. R.F. Turk, J.E. Manno, N.C. Jain, and R.B. Forney. *J. Pharmac., 23,* 190 (1971).
5. J. Rosenfeld, B. Bowins, J. Roberts, J. Perkins, and A.S. Macpherson. *Analytical Chemistry, 46,* 2232 (1974).
6. E.R. Garrett and D.A. Hunt. *Journal of Pharmaceutical Sciences, 63,* 1056 (1974).
7. D. Rosenthal and D.J. Brine. *Forens. Sci., 24,* 282 (1979).

AUTHORS

Anthony S. Wong and Michael W. Orbanosky
California Analytical Laboratories, Inc.
401 N. 16th Street
Sacramento, Calif. 95814
and
Victor C. Reeve and James D. Beede
California Department of Justice
Bureau of Forensic Services
3301 C Street
Sacremento, Calif. 95816

Analysis of Cannabinoids
Research Monograph 42, R. Hawks, Editor
National Institute on Drug Abuse, 1982

The Constituents of Cannabis and the Disposition and Metabolism of Cannabinoids

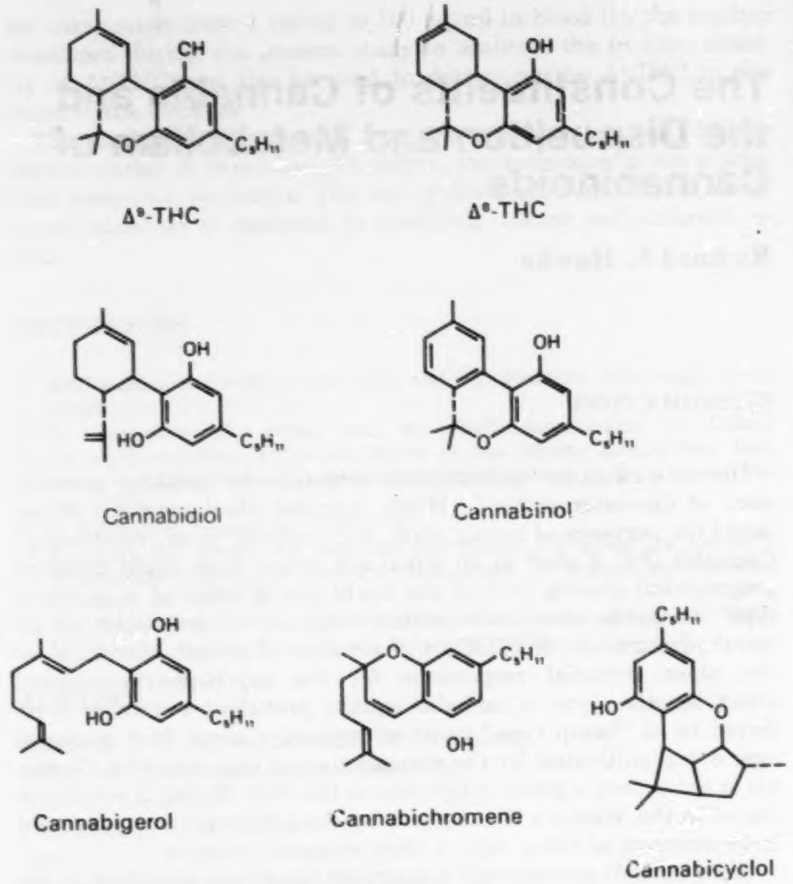
Richard L. Hawks

INTRODUCTION

Humans consume cannabinoids primarily by smoking preparations of Cannabis sativa L. When cannabis plant material is prepared for purposes of intoxication, it is referred to as "marijuana." Cannabis that is used as an intoxicant comes from many different geographical sources around the world and is referred to as "drug type" cannabis because it contains significant quantities of Δ^9-tetrahydrocannabinol (THC), the primary chemical ingredient of the plant material responsible for the psychopharmacological effect. Another type of cannabis equally prevalent worldwide is referred to as "hemp type" because originally, about four centuries ago, it was cultivated for the manufacture of rope material. Cannabis is not in fact a plant indigenous to the New World; it was introduced to the Western Hemisphere by Spanish explorers, who used large amounts of hemp rope in their shipping industry.

More than 60 cannabinoid compounds have been identified in the "drug type" cannabis used illicitly today. Some of the more important ones are illustrated in figure 1. In addition, as in any plant material, there is a large number of other chemical compounds present, several hundred of which have been identified in cannabis (1).

Though cannabis contains many cannabinoid components and other chemical substances, THC is the most prominent psychoactive cannabinoid component, and its concentration determines the "potency" of marijuana. Other cannabinoids may contribute to THC's activity through interactions or direct effects on certain specific pharmacologic measures, but THC is the compound on which



Δ^9-THC

Δ^8-THC

Cannabidiol

Cannabinol

Cannabigerol

Cannabichromene

Cannabicyclol

FIGURE 1. Cannabinoids of primary interest found in Cannabis Sativa.

most studies to date have concentrated. The psychopharmacologic activities of either cannabinol or cannabidiol, for instance, are estimated to be less than 10% as great in man as that of THC itself (2). The predominant factor affecting potency is the genetic stock of the plant. Seeds derived from cannabis of relatively high THC content will produce plants of relatively high potency, other factors being equal. It should also be noted that the relative amounts of cannabinoids in the plant can vary significantly depending on how and where the plant is grown and harvested. Soil, climate, cultivation, and age can have considerable effect on the final "product."

Samples of marijuana from illegal shipments, confiscated between 1979 and 1982 by the Drug Enforcement Administration and analyzed by the NIDA potency monitoring program, averaged above 3.0% THC by weight. Potency has increased significantly since the program started in 1974, when confiscated material was found to contain less than 0.5%. A cannabis preparation called "sinsemilla" has become increasingly available over the last 4 years and is currently averaging about 6% THC. Occasional samples with potencies as high as 10% have been encountered. The potential for ingesting psychologically dysphoric amounts of THC is thus much increased with the availability of these more potent forms of cannabis.

The THC in marijuana is predominantly in the form of Δ^9-THC-2-(and a small amount of 4-)-carboxylic acid (also called "tetrahydrocannabinolic acids" or "THC-acids") (1). These THC-acids are changed rapidly into THC itself when they are subjected to the heat of the burning cigarette. The burning process also causes pyrolytic destruction of a portion of the THC and THC acid in the cigarette. The actual amount of THC delivered in the smoke has been estimated at 20% to 70% in experiments conducted with smoking machines (3). The estimate of 70% is derived from a study in which the cigarette was burned with a continuous draw, and 20% was found when a 5-second puff each minute was used. The latter rate is the standard for tobacco smoking experiments. Marijuana smokers probably fall in between these estimates since they usually inhale longer and more frequently than once a minute for 5 seconds. This assumption is supported by a study which determined that the bioavailability of THC from marijuana cigarettes as measured in blood of human subjects ranged from 8% to 24% (mean 18±6%) in 11 subjects (4). This "bioavailability" is the fraction of THC in the cigarette which reaches the bloodstream. If for instance, 35% of the THC in the cigarette were delivered in the smoke and 50% of this passed through the lung to the blood, a bioavailability of 17.5% would result, which is within the reported range.

For the past 10 years NIDA has supplied cannabis cigarettes for use in animal and human research on marijuana toxicity, pharmacology, pharmacokinetics, and chemistry. The cigarettes are supplied in potencies ranging from 0.5% to 3% by weight. The plant material is grown under controlled conditions, and the material is chemically characterized by quantitative analysis of eight major cannabinoids. The cigarettes are supplied in a form similar in size to unfiltered tobacco cigarettes.

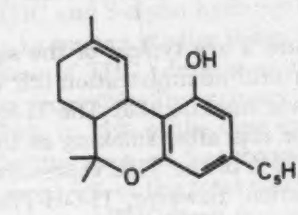
Other THC dosage forms developed by NIDA and made available for research include oral doses of THC in sesame oil contained in gelatine capsules, intravenous solutions of THC which are either suspended in human serum albumen or dissolved in ethanol, and ophthalmic preparations of THC in mineral oil. Each of these dosage forms has its own unique time course of effect and metabolic profile. Even though these dosage forms were developed for use in research directed at consequences of marijuana abuse, they have also served as dosage forms for studies in several areas of therapeutic research. The oral form is under investigation as an antiemetic drug for patients undergoing cancer chemotherapy, and the ophthalmic preparation is being investigated as a treatment to relieve intraocular pressure resulting from glaucoma.

METABOLISM AND DISPOSITION

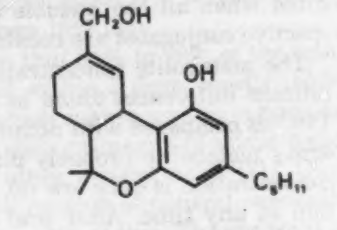
When cannabis is smoked or ingested orally, THC and the many other cannabinoids present undergo extensive metabolism into many oxygenated species. Most of the research on cannabinoid metabolism has been carried out on THC itself. Metabolites of THC which have been characterized and isolated from man (5,6,7) are illustrated in figure 2.

The total number of metabolites resulting from human administration of THC is undoubtedly much greater. In vitro and in vivo animal studies indicate that a large number of hydroxylated and carboxylated metabolites result from the metabolism of THC as well as from the other cannabinoids found in the plant (8-14). Investigators have also shown the presence of fatty acid conjugates of THC (15,16), a variety of 0-glucuronides (17,18), and C-glucuronides (19).

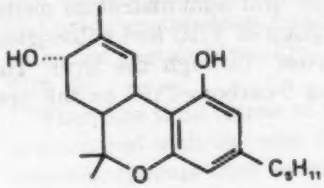
The formation of glucuronide conjugates of THC and its metabolites has been reported to occur on the phenolic position (17), on the carboxylic acid position in 11-nor-Δ^9-THC-9-carboxylic acid (9-carboxy-THC) (18), and directly on the two and four positions of the aromatic ring (19). It is unclear at this time how this conjugation affects excretion, storage, and recirculation of material, but it is clear that a major portion of the cannabinoid metabolites excreted in urine are in the form of glucuronide conjugates (5,18,20). In the case of 9-carboxy-THC, conjugation may occur at both the phenolic and the carboxy position, thus leading to the possibility of three different conjugated species. A recent study has provided the characterization of 12 additional acid metabolites of THC (21). An extensive and complex cannabinoid metabolic profile in man is indi-



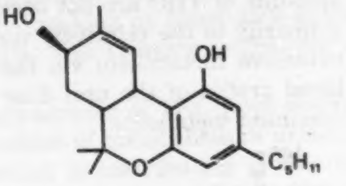
Δ⁹ THC
(active)



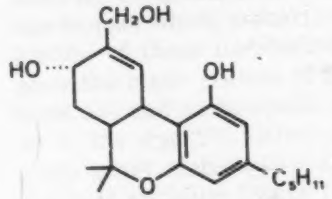
11-Hydroxy-Δ⁹ THC
(active)



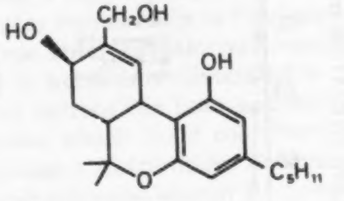
8α-Hydroxy-Δ⁹ THC
(much less active)



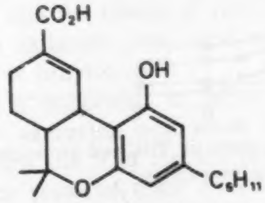
8β-Hydroxy-Δ⁹ THC
(less active)



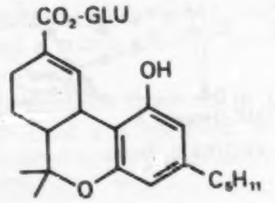
8α, 11-Dihydroxy-Δ⁹ THC



8β, 11-Dihydroxy-Δ⁹ THC



11-nor-Δ⁹ THC-9-
Carboxylic Acid
(inactive)



11-nor-Δ⁹ THC-9-Carboxylic
Acid Glucuronide

FIGURE 2. Metabolites of THC identified in human biological fluids.

cated when all the possible cannabinoid metabolites and their respective conjugates are considered.

The metabolite concentrations in figure 3 are typical of the significant differences found in blood after oral administration (22) of THC as compared with administration via smoking (23). The 11-hydroxy metabolite probably plays a minor role after smoking as the concentration is very low (5), less than 10% of the THC concentration at any time. After oral administration, however, 11-OH-THC may play a more important role as its concentration is relatively higher in blood, often higher than that of THC. Significant amounts of THC are not observed after oral administration owing primarily to the relatively slow absorption of THC and subsequent extensive metabolism via the "first pass" through the liver. The blood profile of the oral dose indicates 9-carboxy-THC as the predominant metabolite.

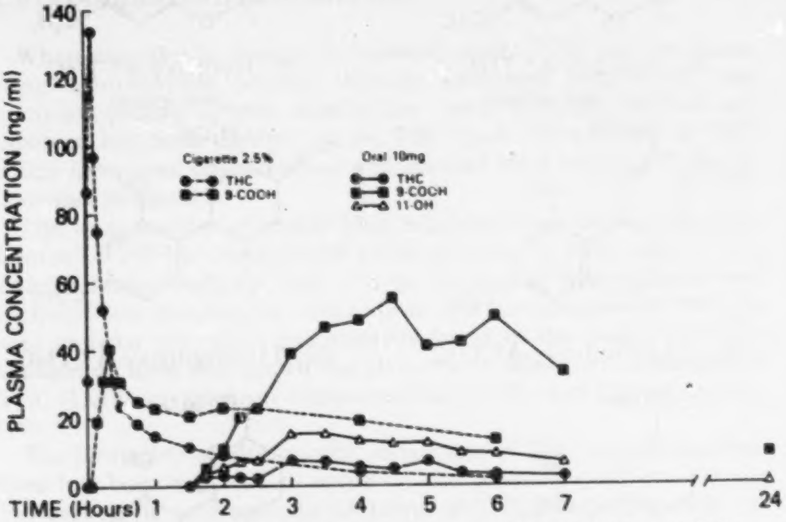


FIGURE 3. Plasma concentration versus time curve for THC and its metabolites after oral and smoked administrations.

Several of the hydroxylated metabolites have been shown to have some activity (24), but 9-carboxy-THC has shown little or no activity when administrated to either man (24) or animal. The only active metabolite known to occur in any significant concentration is 11-hydroxy-Δ^9-THC, which has been shown to have activity equal to or perhaps slightly greater than THC itself when administered alone to man (25). The psychopharmacological activity in man of 8-

beta-hydroxy-THC has been reported as about 4% of that of Δ^9-THC and 8-alpha-hydroxy-THC less than 10% (24).

In human studies it has been shown that 80% to 90% of the total dose of THC administered is excreted within 5 days, mostly (65%) in feces (26). The urinary fraction, which makes up the remaining 18% to 23% of the dose excreted, consists primarily of acidic metabolites such as 9-carboxy-THC and other related acids (21). Only about 5% of the total urinary fraction consists of neutral cannabinoids, and THC itself makes up less than 1% of this. The endpoint of the renal pathway is therefore multi-oxygenated acidic metabolites. The feces fraction consists of approximately equal parts neutral and acidic metabolites, 9-carboxy-THC making up about 29% of the extracted metabolites, THC about 8%, and 11-hydroxy-THC about 21% (5).

When the time course of the disappearance of THC from plasma is compared with the very slow excretion of metabolites in urine, it becomes obvious that some mechanism is contributing to this retarded elimination of material. As mentioned above, 80% to 90% of the THC dose administered IV is accounted for in 5 days, the remainder being eliminated slowly over a longer period of time. One mechanism which probably contributes significantly to the long excretion of these metabolites involves enterohepatic recirculation, since the major portion of IV doses in humans is eliminated in the feces (26) and reabsorption of biliary excreta has been demonstrated in the dog (27). Other mechanisms which could contribute include renal reabsorption and deep-tissue distribution. Because of the lipid solubility (28) of THC, a characteristic shared by many of its metabolites, some portion of the dose is probably trapped in fatty-tissue pools for slow release. Preferential absorption of THC into the fat tissue of rats has been reported, while other tissues such as brain, lung, and muscle absorbed a much smaller proportion of the dose (29).

It is important to define both the mechanisms involved in this slow excretion and what specific metabolites are involved. Even though the acid metabolites are not likely to have psychoactivity, other types of toxic effects cannot be excluded. The characterization of fatty-acid conjugates of THC has led to speculation that species of this type may contribute to storage of cannabinoids in body tissue (15,16). Much research remains to be done to ascertain the chronic toxicity of these or the many other THC metabolites.

The character of the metabolites and their mode of elimination is important since the elimination process is measured in days, not hours, and repeated administration via marijuana smoking likely leads to accumulation of both THC and metabolites and the possibility of subsequent toxic consequences.

Much of the forensic interest in cannabinoid analysis of body fluids has focused on the 9-carboxy-THC metabolite, the principal acid metabolite of THC that has been characterized and synthesized for study. This metabolite is an important species in both blood and urine. As illustrated in figure 3, THC levels which are initially quite high drop very rapidly and cross the rising 9-carboxy-THC curve at approximately 20 minutes. Although this curve is based on a single dose, it does give credence to the hypothesis that a blood analysis which shows similar concentrations for both THC and 9-carboxy-THC could be an indication of very recent administration of marijuana and perhaps of a high probability that the subject is in an intoxicated state. This figure also illustrates that, because of the rapid decrease in the concentration of THC in blood, analytical methodology less sensitive than 1 or 2 nanograms per milliliter is not likely to detect the presence of THC after several hours except perhaps in a heavy chronic smoker. The curve for 9-carboxy-THC in this figure shows a considerably longer time course and therefore provides a better indicator of previous smoking.

Urine metabolite assays which have recently become available on the commercial market have made cannabinoid detection available to a much broader spectrum of laboratories including those associated with the armed forces, with private industry, and penal institutions. These methods include the SYVA Co. EMIT system and the Roche Diagnostics ABUSCREEN RIA system. The analysis of urine using these types of assays provides an indication of past use of marijuana. Due to the relatively long detection period of cannabinoid metabolites in urine, this 'past use' cannot be more specifically pinpointed than between 1 hour and 1 week. This makes any urine analysis, regardless of the levels detected, inadequate for prediction of intoxication or specific time of use.

As mentioned above, a blood analysis does have the potential for providing information relevant to intoxication. Blood samples are, however, difficult to obtain for many forensic purposes because of their invasive nature. Saliva and breath are much easier samples to acquire for drug detection and both have been investigated for the presence of marijuana components after smoking. The use of TLC methods for detection of THC in saliva has been suggested by several authors (30-34).

A study in humans using radiolabeled THC administered by intravenous injection (35) failed to detect any radioactivity in saliva samples. This suggests that neither THC or its metabolites pass into the saliva or lung from the blood and that cannabinoids detected in these biological matrices are sequestered during the smoking process.

In a recent study, an RIA specific for THC was used (36) to analyze a set of saliva samples (37) from subjects who smoked marijuana cigarettes containing cannabis of approximately 2% potency. Mixed saliva samples were taken from these subjects over a 24-hour period. The concentration of sequestered THC in these samples, illustrated in figure 4, is subject to considerably more fluctuation over time than plasma levels due to the inherent variability of mixed saliva concentration and to the difficulty in precisely aliquoting from a mixed saliva sample. It is clear, however, that significant levels of THC remain in saliva for several hours after smoking marijuana. During the first hour, in the absence of food intake, some levels exceeded 100 ng/ml and levels above 5 ng/ml

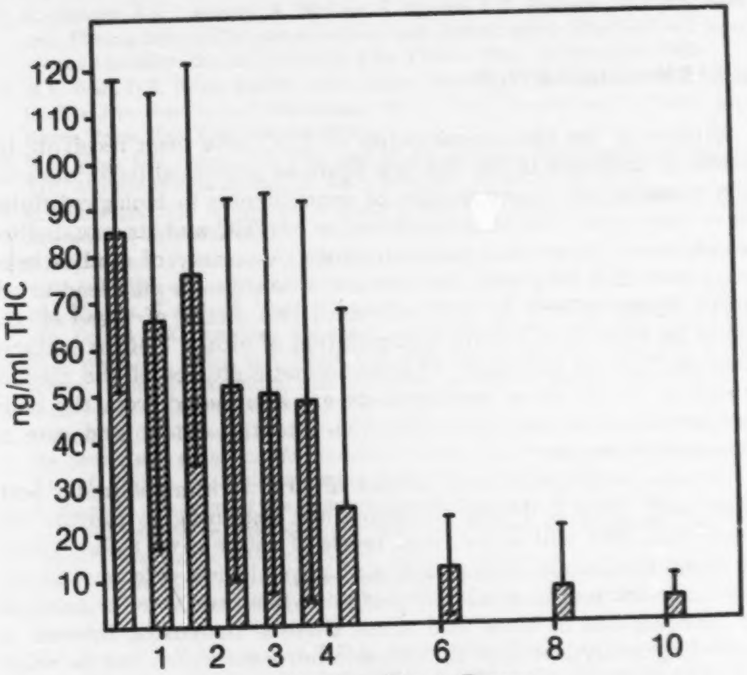


FIGURE 4. Human saliva levels after smoking a single marijuana cigarette containing 18 mg THC. N=8 at 20 min., N=7 at 60 min., N=5 for others. Variability expressed as standard deviation.

were still detectable in most samples beyond 8 hours even though normal food and liquid intake was resumed after 3 hours.

These results give further indication that saliva could be a useful fluid for presumptive detection of recent use. Even though no quantitative correlation can be made between saliva levels and intoxication since only sequested THC is being detected, the capacity to reduce the "past use" time frame from weeks to hours could have useful research and forensic applications.

The use of breath as a screening medium has been investigated using solvent and cryogenic traps with subsequent analysis by HPLC/MS (38) and also using solid matrix traps followed by conventional RIA (39). The levels in breath appear to be lower than those found in saliva after smoking, and use of such a medium for detection would consequently require relatively more sensitivity in the assay method as well as a more complex means of sample collection.

PHARMACOKINETICS

Studies of the pharmacokinetics of THC have been receiving increasing emphasis in the last few years as analytical techniques for the quantitative determination of cannabinoids in biological fluids have improved. The pharmacokinetics of THC and its metabolites is important to several research areas. A variety of studies have suggested that long-term, chronic use of marijuana may lead to adverse health effects in the individual (40). Some of these effects could be related to a toxic accumulation of either THC or metabolites of THC in the body. Pharmacokinetic studies of the mechanisms by which these cannabinoids are eliminated from the body are important in understanding the potential extent and rate of this accumulation.

Detailed pharmacokinetic studies of THC in humans under both acute and chronic dosing schedules are beginning to provide the basic data that will allow blood levels of active drug to be related to psychotomimetic, behavioral, and physiological effects (4,23,26). With the increasing availability of analytical methods for analysis of cannabinoids in blood and urine, there is increasing interest in providing analytical data for forensic purposes which can be related to the intoxication of individuals involved in accidents, as well as individuals who are legally liable for drug-taking behavior in the armed forces, on probation, or in industry. It is important to understand the kinetic mechanisms involved with THC and other cannabinoids in order to relate these forensic questions to the meaning of a given blood level, particularly in cases where intoxication is a factor. The availability of more sensitive and more accessible analytical technology for the quantitative analysis of marijuana components in body fluids will play a key role in these efforts.

REFERENCES

1. C.T. Turner, M.A. Elsohly, and E.G. Boeren. Constituents of Cannabis Sativa L. XVII. A review of the natural constituents. *J. Natural Products, 43,* 169-234 (1980).
2. M. Perez-Reyes, M.C. Timmons, K.H. Davis, and M.E. Wall. A comparison of the pharmacological activity in man of intravenously administered delta-9-THC, cannabinol and cannabidiol. *Experientia, 29,* 1368-1369 (1973).
3. K.H. Davis, Research Triangle Institute, private communication.
4. A. Ohlsson, J-E. Lindgren, A. Wahlen, S. Agurell, L.E. Hollister, and H.K. Gillispie. Plasma delta-9-THC concentrations and clinical effects after oral and intravenous administration and smoking. *Clin. Pharm. Ther., 28,* 409-1413 (1980).
5. M.E. Wall, D.R. Brine, and M. Perez-Reyes. Metabolism of cannabinoids in man. In *The Pharmacology of Marijuana, Vol. 1,* M.C. Braude and S. Szara, eds., Raven Press, New York, 93-116 (1976).
6. M.E. Wall, D.R. Brine, C.G. Pitt, and M. Perez-Reyes. Identification of delta-9-THC and metabolites in man. *J. Am. Chem. Soc., 94,* 8579-8581 (1972).
7. L. Lemberger, S.D. Silberstein, J. Axelrod, and I.J. Kopin. Delta-9-THC in man. *Science, 170,* 1320-1322 (1970).
8. S.H. Burstein, F. Menezes, E. Williamson, and R. Mechoulam. Metabolism of delta-1(6)-THC, an active marihuana constituent. *Nature, 225,* 87-88 (1979).
9. I.M. Nilsson, S. Agurell, J.L.G. Nilsson, A. Ohlsson, F. Sandberg, and M. Wahlqvist. Delta-1-THC: structure of a major metabolite. *Science, 168,* 1228-1229 (1970).
10. M.E. Wall, D.R. Brine, G.A. Brine, C.G. Pitt, R.I. Freudenthal, and H.D. Christensen. Isolation, structure, and biological activity of several metabolites of delta-9-THC. *J. Am. Chem. Soc., 92,* 3466-3468 (1970).
11. S. Agurell, I.M. Nilsson, M. Widman. Cannabinoids: metabolites hydroxylated in the pentyl side chain. In *Marihuana Chemistry, Biochemistry and Cellular Effects,* G. Nahas, ed., Springer-Verlag, New York, 141-158 (1976).
12. S. Burstein. A survey of the metabolic transformations of delta-1-THC. In *Cannabinoid Analysis in Physiological Fluids.* J.A. Vinson, ed., American Chemical Society, Washington, D.C., 1-12 (1979).
13. M.E. Wall and D.R. Brine. Applications of mass spectrometry in cannabinoid research. In *Marihuana: Biological Effects,* G. Nahas and W.D.M. Paton, eds., Pergamon Press, London, 15-43 (1979).
14. D.J. Harvey, B.R. Martin, and W.D.M. Paton. Identification and measurement of cannabinoids and their in vivo metabolites in liver by gas chromatography-mass spectrometry. In *Marihuana: Biological Effects,* G.G. Nahas and W.D.M. Paton, eds., Pergamon Press, London, 45-62 (1979).
15. E.G. Leighty, A.F. Fentiman, and R.E. Foltz. Long retained metabolites of delta-9-THC identified as novel fatty acid conjugates. *Res. Commun. Chem. Pathol. Pharmacol., 14,* 13-28 (1976).

16. W. Yisak, S. Agurell, J-E. Lingren, and M. Widman. In vivo metabolites of cannabinol identified as fatty acid conjugates. *J. Pharm. Pharmacol., 30,* 462-463 (1978).
17. S. Pallante, M.A. Lyle, and C. Fenselau. Synthesis and characterization of glucuronides of 5'-hydroxy-delta-9-THC and 11-hydroxy-delta-9-THC. *Drug Metab. Dispos., 6,* 389-395 (1978).
18. P.L. Williams and A.C. Moffat. Identification in human urine of delta-9-THC-11-oic acid glucuronide: A THC metabolite. *J. Pharm. Pharmacol., 32,* 445-448 (1980).
19. S. Levy, B. Yagen, and R. Mechoulam. Identification of a C-glucuronide of delta-6-tetrahydrocannabinol as a mouse liver conjugate in vivo. *Science, 200,* 1391-1392 (1978).
20. S.L. Kanter, L.E. Hollister, and J.U. Zamora. Marijuana metabolites in urine of man XI. Detection of unconjugated and conjugated delta-9-THC-11-oic acid by thin layer chromatography. *J. Chromatogr., 235,* 507-512 (1982).
21. M.M. Halldin, S. Carlsson, S.L. Kanter, M. Widman, and S. Agurell. Urinary metabolites of Δ^1-tetrahydrocannabinol in man. Arzneimittelforschung, in press.
22. H. Gross, M.H. Ebert, V.B. Faden, S.C. Goldberg, W.H. Kaye, E.D. Caine, R.L. Hawks, and N. Zinberg. A double-blind trial of delta-9-tetrahydrocannabinol in primary anorexia nervosa. Submitted for publication, 1982.
23. M. Perez-Reyes, S. DiGuiseppi, K.H. Davis, V.H. Schindler, and C.E. Cook. Comparison of effects of marihuana cigarettes of three different potencies. *Clin. Pharmacol. Ther., 31,* 617-624 (1982).
24. M. Perez-Reyes, M.D. Timmons, M.A. Lipton, H.D. Christensen, and K.H. Davis. A comparison of the pharmacological activity of delta-9-THC and its monohydroxylated metabolites in man. *Experientia, 29,* 1009-1010 (1973).
25. M. Perez-Reyes, M.C. Timmons, M.A. Lipton, K.H. Davis, and M.E. Wall. Intravenous injection in man of delta-9-THC and 11-OH-delta-9-THC, *Science, 177,* 633-635 (1972).
26. A.C. Hunt and R.T. Jones. Tolerance and disposition of THC in man. *J. Pharm. Exp. Ther., 215,* 35-44 (1980).
27. E.R. Garrett and C.A. Hunt. Pharmacokinetics of delta-9-THC in dogs. *J. Pharm. Sci., 66,* 395-407 (1977).
28. E.R. Garrett and C.A. Hunt. Physicochemical properties, solubility, and protein binding of delta-9-THC. *J. Pharm. Sci., 63,* 1056-1064 (1974).
29. D.S. Kreuz and J. Axelrod. Delta-9-tetrahydrocannabinol: localization in body fat. *Science, 179,* 391-393 (1973).
30. I.S. Forrest, D.E. Green, S.E. Rose, G.C. Skinner, and D.M. Torres. Flourescent-labeled cannabinoids. *Res. Commun. Chem. Pathol. Pharmacol., 2,* 787-792 (1971).
31. A.P. Melikian and I.S. Forrest. Dansyl derivatives of delta-9- and delta-8-tetrahydrocannabinols. *J. Pharm Sci., 62,* 1025-1026 (1973).
32. W.W. Just, G. Werner, and M. Weichmann. Bestimmung von delta-1- and delta-1(6) Tetrahydrocannabinol in Blut, Urin und Speichel von Haschisch-Rauchern. *Naturwiss., 59,* 222-223 (1972).
33. F. Friedrich-Fiechtl, G. Spiteller, W.W. Just, G. Werner, and M. Weichmann. Au Nachweis und Identifizierung von Tetrahydrocannabinol in Biologischen Flussighkeiten. *Naturwiss., 60,* 207-208 (1973).
34. W.W. Just, N. Filipovic, and G. Werner. Detection of delta-9-THC in saliva of men by means of thin-layer chromatography and mass-spectrometry. *J. Chromatogr., 96,* 189-194 (1974).
35. M. Perez-Reyes, University of North Carolina, personal communication.

36. C.E. Cook, Research Triangle Institute, NC, performed under NIDA contract 271-80-3705.
37. H. Moskowitz, Southern California Research Institite, CA, samples collected under NIDA contract 271-76-3316.
38. J.L. Valentine, P.J. Bryant, P.L. Gutshall, O.H.M. Gan, and H.C. Niu. Detection of delta-9-THC in human breath following marijuana smoking. *Anal. Letters, 12* (B8) 867-880 (1979).
39. J.R. Soares, J.D. Grant, and S.J. Gross. Significant developments in radioimmune methods applied to delta-9-THC and its 9-substituted metabolites. This volume.
40. *Marijuana and Health.* Report of a study by a Committee of the Institute of Medicine, A.S. Relman, chairman. National Academy Press, Washington, D.C., 1982.

AUTHOR

Richard L. Hawks
Research Technology Branch
Division of Research
National Institute on Drug Abuse
Rockville, Maryland 20857

National
Institute on
Drug
Abuse

Research

monograph series

While limited supplies last, single copies of the monographs may be obtained free of charge from the National Clearinghouse for Drug Abuse Information (NCDAI). Please contact NCDAI also for information about availability of coming issues and other publications of the National Institute on Drug Abuse relevant to drug abuse research.

Additional copies may be purchased from the U.S. Government Printing Office (GPO) and/or the National Technical Information Service (NTIS) as indicated. NTIS prices are for paper copy. Microfiche copies, at $4.00, are also available from NTIS. Prices from either source are subject to change. Addresses are:

NCDAI
National Clearinghouse for Drug Abuse Information
Room 10A-53
5600 Fishers Lane
Rockville, Maryland 20857

GPO
Superintendent of Documents
U.S. Government Printing Office
Washington, D.C. 20402

NTIS
National Technical Information Service
U.S. Department of Commerce
Springfield, Virginia 22161

1 FINDINGS OF DRUG ABUSE RESEARCH. Not available from NCDAI.
Vol. 1: GPO out of stock NTIS PB #272 867/AS $28.50
Vol. 2: GPO out of stock NTIS PB #272 868/AS $28.50

2 OPERATIONAL DEFINITIONS IN SOCIO-BEHAVIORAL DRUG USE RESEARCH 1975. Jack Elinson, Ph.D., and David Nurco, Ph.D., eds. Not available from NCDAI.
GPO out of stock NTIS PB #246 338/AS $15

3 AMINERGIC HYPOTHESES OF BEHAVIOR: REALITY OR CLICHE? Bruce J. Bernard, Ph.D., ed.

GPO Stock #017-024-00486-3 $6.50 NTIS PB #246 687/AS $15

4 NARCOTIC ANTAGONISTS: THE SEARCH FOR LONG-ACTING PREPARATIONS. Robert Willette, Ph.D., ed.

GPO out of stock NTIS PB #247 096/AS $7.50

5 YOUNG MEN AND DRUGS: A NATIONWIDE SURVEY. John A. O'Donnell, Ph.D., et al. Not available from NCDAI.

GPO Stock #017-024-00511-8 $6.50 NTIS PB #247 446/AS $15

6 EFFECTS OF LABELING THE "DRUG ABUSER": AN INQUIRY. Jay R. Williams, Ph.D. Not available from NCDAI.

GPO Stock #017-024-00512-6 $4.75 NTIS PB #249 092/AS $7.50

7 CANNABINOID ASSAYS IN HUMANS. Robert Willette, Ph.D., ed.

GPO Stock #017-024-00510-0 $6.00 NTIS PB #251 905/AS $13.50

8 Rx: 3x/WEEK LAAM - ALTERNATIVE TO METHADONE. Jack Blaine, M.D., and Pierre Renault, M.D., eds.

Not available from GPO NTIS PB #253 763/AS $13.50

9 NARCOTIC ANTAGONISTS: NALTREXONE PROGRESS REPORT. Demetrios Julius, M.D., and Pierre Renault, M.D., eds.

GPO Stock #017-024-00521-5 $7.00 NTIS PB #255 833/AS $16.50

10 EPIDEMIOLOGY OF DRUG ABUSE: CURRENT ISSUES. Louise G. Richards, Ph.D., and Louise B. Blevens, eds. *Examines methodological issues in surveys and data collection.* Not available from NCDAI.

GPO Stock #017-024-00571-1 $6.50 NTIS PB #266 691/AS $21

11 DRUGS AND DRIVING. Robert Willette, Ph.D., ed. *Reviews research on effects of drugs on psychomotor performance, focusing on measures of impairment by different drugs at various levels.*Not available from NCDAI.

GPO Stock #017-024-00576-2 $5.50 NTIS PB #269 602/AS $15

12 PSYCHODYNAMICS OF DRUG DEPENDENCE. Jack D. Blaine, M.D., and Demetrios A. Julius, M.D., eds. *Theoretical and clinical papers concerned with the intrapsychic determinants of drug addiction.*

GPO Stock #017-024-00642-4 $5.50 NTIS PB #276 084/AS $16.50

13 COCAINE: 1977. Robert C. Petersen, Ph.D., and Richard C. Stillman, M.D., eds. *Reports the extent and limits of current knowledge about cocaine, its use and misuse.*

GPO Stock #017-024-00592-4 $6.00 NTIS PB #269 175/AS $18

14 MARIHUANA RESEARCH FINDINGS: 1976. Robert C. Petersen, Ph.D., ed. *Technical papers on which the 6th Marihuana and Health report to Congress was based.*

GPO out of stock NTIS PB #171 279/AS $21

15 REVIEW OF INHALANTS: EUPHORIA TO DYSFUNCTION. Charles Wm. Sharp, Ph.D., and Mary Lee Brehm, Ph.D., eds. *Review of inhalant abuse, including an extensive bibliography.*

GPO Stock #017-024-00650-5 $7.50 NTIS PB #275 798/AS $27

16 THE EPIDEMIOLOGY OF HEROIN AND OTHER NARCOTICS. Joan Dunne Rittenhouse, Ph.D., ed. *Task Force report on research technologies and implications for studying heroin-narcotic use.*

GPO Stock #017-024-00690-4 $6.50 NTIS PB #276 357/AS $19.50

17 RESEARCH ON SMOKING BEHAVIOR. Murray E. Jarvik, M.D., Ph.D., et al., eds. *Includes epidemiology, etiology, consequences of use, and approaches to behavioral change. From a NIDA-supported UCLA conference.*

GPO Stock #017-024-00694-7 $7.50 NTIS PB #276 353/AS $28.50

18 BEHAVIORAL TOLERANCE: RESEARCH AND TREATMENT IMPLICATIONS. Norman A. Krasnegor, Ph.D., ed. *Theoretical and empirical studies of nonpharmacologic factors in development of drug tolerance.*
GPO Stock #017-024-00699-8 $5.50 NTIS PB #276 337/AS $15

19 THE INTERNATIONAL CHALLENGE OF DRUG ABUSE. Robert C. Petersen, Ph.D., ed. *Papers from the VI World Congress of Psychiatry which deal with drug issues of particular interest worldwide.*
GPO Stock #017-024-00822-2 $7.50 NTIS PB #293 807/AS $27

20 SELF-ADMINISTRATION OF ABUSED SUBSTANCES: METHODS FOR STUDY. Norman A. Krasnegor, Ph.D., ed. *Techniques used to study basic processes underlying abuse of drugs, ethanol, food, and tobacco.*
GPO Stock #017-024-00794-3 $6.50 NTIS PB #288 471/AS $21

21 PHENCYCLIDINE (PCP) ABUSE: AN APPRAISAL. Robert C. Petersen, Ph.D., and Richard C. Stillman, M.D., eds. *Pioneering volume for clinicians and researchers assessing what is known about the problem of PCP abuse.*
GPO Stock #017-024-00785-4 $7.00 NTIS PB #288 472/AS $24

22 QUASAR: QUANTITATIVE STRUCTURE ACTIVITY RELATIONSHIPS OF ANALGESICS, NARCOTIC ANTAGONISTS, AND HALLUCINOGENS. Gene Barnett, Ph.D.; Milan Trsic, Ph.D.; and Robert Willette, Ph.D.; eds. *Reports from an interdisciplinary conference on the molecular nature of drug-receptor interactions.* Not available from NCDAI.
GPO Stock #017-024-00786-2 $8.00 NTIS PB #292 265/AS $34.50

23 CIGARETTE SMOKING AS A DEPENDENCE PROCESS. Norman A. Krasnegor, Ph.D., ed. *Discusses factors involved in the onset, maintenance, and cessation of the cigarette smoking habit. Includes an agenda for future research.*
GPO Stock #017-024-00895-8 $6.00 NTIS PB #297 721/AS $18

24 SYNTHETIC ESTIMATES FOR SMALL AREAS: STATISTICAL WORKSHOP PAPERS AND DISCUSSION. Joseph Steinberg, ed. *Papers from a workshop cosponsored by NIDA and the National Center for Health Statistics on a class of statistical approaches that yield needed estimates of data for States and local areas.* Not available from NCDAI.
GPO Stock #017-024-00911-3 $8.00 NTIS PB #299 009/AS $22.50

25 BEHAVIORAL ANALYSIS AND TREATMENT OF SUBSTANCE ABUSE. Norman A. Krasnegor, Ph.D., ed. *Papers present commonalities and implications for treatment of dependency on drugs, ethanol, food, and tobacco.*
GPO Stock #017-024-00939-3 $5.00 NTIS PB #80-112428 $21

26 THE BEHAVIORAL ASPECTS OF SMOKING. Norman A. Krasnegor, Ph.D., ed. *Reprint of the behavioral section of the 1979 Report of the Surgeon General on Smoking and Health; introduction by editor.*
GPO out of stock NTIS PB #80-118755 $16.50

27 PROBLEMS OF DRUG DEPENDENCE, 1979: PROCEEDINGS OF THE 41ST ANNUAL SCIENTIFIC MEETING, THE COMMITTEE ON PROBLEMS OF DRUG DEPENDENCE, INC. Louis S. Harris, Ph.D., ed. Not available from NCDAI.
GPO Stock #017-024-00981-4 $9 NTIS PB #80-175482 $36

28 NARCOTIC ANTAGONISTS: NALTREXONE PHARMACOCHEMISTRY AND SUSTAINED-RELEASE PREPARATIONS. Robert Willette, Ph.D., and Gene Barnett, *Ph.D., eds. Papers report research on sustained-release and long-acting devices for use with the narcotic antagonist naltrexone.*
GPO Stock #017-024-01081-2 $7.00 NTIS PB #81-238875 $22.50

29 DRUG ABUSE DEATHS IN NINE CITIES: A SURVEY REPORT. Louis A. Gottschalk, M.D., et al. *Epidemiologic study providing data on drug-involved deaths and procedures for their investigations.*Not available from NCDAI.
GPO Stock #017-024-00982-2 $6.50 NTIS PB #80-178882 $16.50

30 THEORIES ON DRUG ABUSE: SELECTED CONTEMPORARY PERSPECTIVES. Dan J. Lettieri, Ph.D.; Mollie Sayers; and Helen Wallenstein Pearson, eds. *Volume presents summaries of the major contemporary theories of drug abuse by each of 43 leading theorists.*

GPO Stock #017-024-00997-1 $10.00 Not available from NTIS

31 MARIJUANA RESEARCH FINDINGS: 1980. Robert C. Petersen, Ph.D., ed. *The text of the 8th Marijuana and Health report to Congress and the background scientific papers on which this summary report was based.*

GPO out of stock NTIS PB #80-215171 $19.50

32 GC/MS ASSAYS FOR ABUSED DRUGS IN BODY FLUIDS. Rodger L. Foltz, Ph.D.; Ailison F. Fentiman, Jr., Ph.D.; and Ruth B. Foltz. *A collection of methods for the quantitative analysis of several important drugs of abuse by the technique of gas chromatography-mass spectrometry.*

GPO Stock #017-024-01015-4 $6.00 NTIS PB #81-133746 $18

33 BENZODIAZEPINES: A REVIEW OF RESEARCH RESULTS, 1980. Stephen I. Szara, M.D., D.Sc., and Jacqueline P. Ludford, M.S., eds. *A RAUS (Research Analysis and Utilization System) Review Report on the abuse liability of the benzodiazepine "tranquilizers."*

GPO Stock #017-024-01108-8 $5.00 NTIS PB #82-139106 $12.00

34 PROBLEMS OF DRUG DEPENDENCE, 1980: PROCEEDINGS OF THE 42ND ANNUAL SCIENTIFIC MEETING, THE COMMITTEE ON PROBLEMS OF DRUG DEPENDENCE, INC. Louis S. Harris, Ph.D., ed. *Comprehensive assemblage of ongoing research on drug abuse, addiction, and new compounds.*

GPO Stock #017-024-01061-8 $8 NTIS PB #81-194847 $33

35 DEMOGRAPHIC TRENDS AND DRUG ABUSE, 1980-1995. Louise G. Richards, Ph.D., ed. *Estimates of probable extent and nature of nonmedical drug use, 1980-1995, based on age structure and other characteristics of U.S. population.*

GPO Stock #017-024-01087-1 $4.50. NTIS PB #82-103417 $12.00

36 NEW APPROACHES TO TREATMENT OF CHRONIC PAIN: A REVIEW OF MULTIDISCIPLINARY PAIN CLINICS AND PAIN CENTERS. Lorenz K.Y. Ng, M.D., ed. *A sharing of ideas among active practitioners in the treatment of pain.*

GPO Stock #017-024-01082-1 $5.50. NTIS PB #81-240913 $18.00

37 BEHAVIORAL PHARMACOLOGY OF HUMAN DRUG DEPENDENCE. Travis Thompson, Ph.D., and Chris E. Johanson, Ph.D., eds. *Presents a growing body of data, systematically derived, on the behavioral mechanisms involved in use and abuse of drugs.*

GPO Stock #017-024-01109-6 $6.50 NTIS PB #82-136961 $24.00

38 DRUG ABUSE AND THE AMERICAN ADOLESCENT. Dan J. Lettieri, Ph.D., and Jacqueline P. Ludford, M.S., eds. *A RAUS Review Report, emphasizing use of marijuana: epidemiology, socio-demographic and personality factors, family and peer influence, delinquency, and biomedical consequences.*

GPO Stock #017-024-01107-0 $4.50 NTIS PB #82-148198 $13.50

39 YOUNG MEN AND DRUGS IN MANHATTAN: A CAUSAL ANALYSIS. Richard R. Clayton, Ph.D., and Harwin L. Voss, Ph.D. *Examines the etiology and natural history of drug use, with special focus on heroin. Includes a Lifetime Drug Use Index.*

GPO Stock #017-024-01097-9 $5.50 NTIS PB #82-147372 $18.00

40 ADOLESCENT MARIJUANA ABUSERS AND THEIR FAMILIES. Herbert Hendin, M.D., Ann Pollinger, Ph.D., Richard Ulman, Ph.D., and Arthur Carr, Ph.D. *A psychodynamic study of adolescents involved in heavy marijuana use, to determine what interaction between family and adolescent gives rise to drug abuse.*

GPO Stock #017-024-01098-7 $4.50 NTIS PB #82-133117 $12.00

41 PROBLEMS OF DRUG DEPENDENCE, 1981: PROCEEDINGS OF THE 43RD ANNUAL SCIENTIFIC MEETING, THE COMMITTEE ON PROBLEMS OF DRUG DEPENDENCE, INC. Louis S. Harris, Ph.D., ed. *A broad review of current research. Includes treatment issues; chemistry and pharmacology of abused drugs; efficacy and dependence liability of new compounds.*

Not available from GPO NTIS PB #82-190760 $40.50

U.S. GOVERNMENT PRINTING OFFICE : 1982 O - 388-952

DEPARTMENT OF
HEALTH AND HUMAN SERVICES
PUBLIC HEALTH SERVICE

ALCOHOL, DRUG ABUSE, AND
MENTAL HEALTH ADMINISTRATION
5600 FISHERS LANE
ROCKVILLE, MARYLAND 20857

OFFICIAL BUSINESS
Penalty for private use, $300

POSTAGE AND FEES PAID
U.S. DEPARTMENT OF H.H.S.
HHS 396

THIRD CLASS
BULK RATE

U.S.MAIL

NOTICE OF MAILING CHANGE

☐ Check here if you wish to discontinue receiving this type of publication.

☐ Check here if your address has changed and you wish to continue receiving this type of publication. (Be sure to furnish your complete address including zip code.)

Tear off cover with address label and publication number still affixed and send to:

Alcohol, Drug Abuse, and Mental Health Administration Publications
PHS Printing and Reproduction Management Branch
5600 Fishers Lane (Rm. 5B-19)
Rockville, Maryland 20857

DHHS Publication No. (ADM) 82-1212
Printed 1982